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DISTURBANCES OF FUNCTION INDUCED BY LEWISITE  
(2-CHLORVINYLDICHLORARSINE) By G R CAMERON,  
F C COURTICE, and R H D SHORT From the Experimental  
Station, Porton, Wilts

(Received for publication 16th January 1946)

THE pathological changes produced by lewisite and allied compounds have been described by Cameron, Carleton, and Short [1946] Lewisite, when applied to the skin of animals, produces locally a central area of necrosis surrounded by a large area of œdema In addition to this local lesion the absorbed lewisite oxide circulating in the blood-stream causes general systemic effects It is the functional disturbances resulting from the capillary damage and the consequent upset of water balance that have been investigated here

Four main groups of experiments have been performed

- (i) The blood and circulatory changes during the period of "shock" which results from the capillary damage and formation of œdema
- (ii) The flow and composition of lymph from various ducts of the body
- (iii) The renal disturbance secondary to the anhydræmia
- (iv) The late anæmia which follows the period of "shock"

### METHODS

*General*—Rabbits, goats, and dogs have been used in these experiments All the animals, except where otherwise stated, were un-anæsthetized In the majority of experiments the application of pure liquid lewisite to the clipped skin of the animals was investigated, while in some cases the effects of intravenous injection of the water soluble lewisite oxide,  $\beta$ -chlorvinylarsenoxide, were also studied

The investigation of the blood and circulatory changes entailed the measurement of the plasma volume, hæmatocrit, hæmoglobin per cent, red-cell count, plasma protein concentration, plasma N P N and urea, blood pressure and body temperature In order to investigate further the capillary damage and loss of fluid, the lymph drainage of the body was studied In these experiments lymph was collected from ducts draining the local lesion and also draining tissues remote from the local lesion in dogs following skin contamination with lewisite Experiments with lymph drainage of the body were also performed in which lewisite

oxide was injected intravenously, in which case there is no local site of contamination

As a result of fluid loss due to oedema formation giving rise to circulatory changes, disturbances of renal function were investigated. These investigations were made with rabbits in chronic experiments and with anaesthetized dogs in acute experiments. To collect 24-hour samples of urine from the rabbit, the animal was kept in a metabolism cage and any urine passed during the day was collected. In addition each rabbit (all males used) was catheterized at the same time each morning, so that the total 24-hour sample of urine was accurately measured. In acute experiments with dogs, the male was used and a catheter passed into the bladder. The bladder could thus be emptied at definite time intervals.

*Analytical Methods* —The detailed methods used are as follows —

- (i) *Blood Volume* —The blue dye, Evans Blue or T-1824, has been used. The method of applying this is described by Courtice [1943]
- (ii) *Hæmoglobin per cent* —Haldane hæmoglobinometer
- (iii) *Red-Cell Count* —Thoma hæmocytometer
- (iv) *Plasma and Lymph Proteins* —The micro Kjeldahl digestion and Nesslerization method of Wong [1923] was employed to determine the protein concentration. Albumin and globulin were determined by precipitating the globulin with 22 per cent sodium sulphate at 38° C [Howe 1921], and estimating the albumin in the filtrate by micro Kjeldahl digestion and Nesslerization.
- (v) *Non-Protein Nitrogen (N P N)* —This has been determined by micro-Kjeldahl digestion and Nesslerization after precipitating the proteins with trichloroacetic acid.
- (vi) *Plasma Urea* —Determined colorimetrically after digestion with urease and Nesslerization [cf Harrison, 1937]
- (vii) *Urine Urea* —Manometric determination using the van Slyke machine [Peters and van Slyke, 1932]
- (viii) *Body Temperature* —Measured rectally with clinical thermometer
- (ix) *Blood Pressure* —In acute experiments with anaesthetized animals, blood pressure was determined by cannulating an artery (femoral or carotid) and recording on a kymograph. In unanaesthetized rabbits the blood pressure was determined by the method described by Grant and Rothschild [1934]

Blood samples were taken from the ear veins in rabbits and from the jugular veins in goats and dogs. Potassium oxalate was used to prevent coagulation of the blood, and dry heparin introduced into the cannula at intervals to prevent coagulation of the lymph.

## RESULTS

1 *Lewisite Shock*

After the skin application of small amounts of lewisite, animals were noticed to be in a state of collapse with cold extremities and collapsed peripheral circulation. These observations of the general attitude of the animal together with the large amount of fluid loss as a result of the cedema led to an investigation of the general systemic effects of lewisite and in particular to a study of the changes in the blood which might be affected by the local loss of fluid.

Experiments were, therefore, carried out in which were determined the blood volume, plasma protein concentration, non-protein nitrogen,

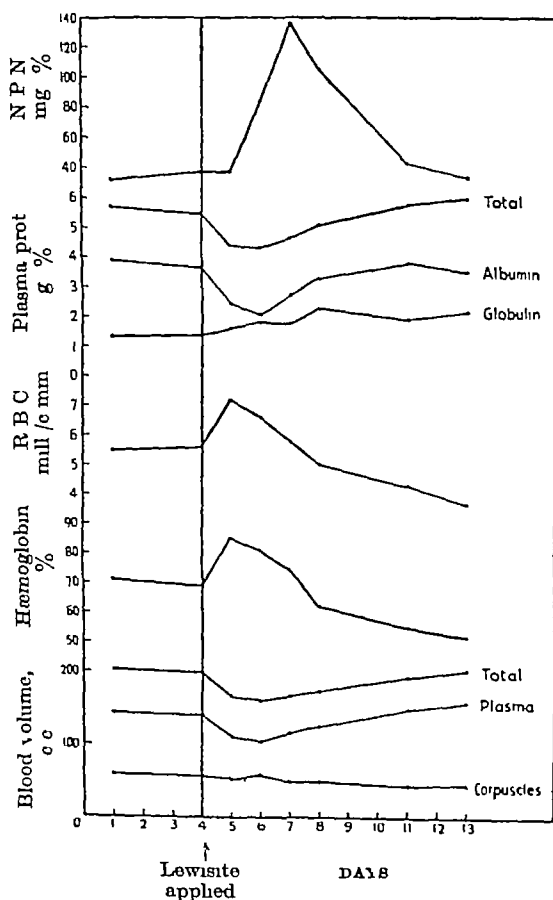


FIG 1—The effects of skin application of lewisite, 4 mg/kg, on blood volume, haemoglobin per cent, red-cell count, plasma protein per cent, and plasma N.P.N. in rabbits. Mean of four experiments.

haemoglobin per cent, and red-cell count of the blood. These determinations were made on unanæsthetized animals, daily for two or more days before the application of lewisite, and then daily afterwards until the animal either died or recovered. Two groups of experiments were performed. In the first the effects of skin application of lewisite, and in the second the effects of the intravenous injection of the water soluble lewisite oxide, were investigated.

(i) *Skin Application of Lewisite*—Both rabbits and goats were used in these experiments. Rabbits were given 4 mg/kg body-weight and goats 10 to 16 mg/kg.

(a) *Rabbits*—The general effects on the blood can be seen in fig. 1. The results in this figure are the averages obtained from experiments on four rabbits, each individual animal behaving in an almost identical manner. All these animals survived. In Table I are similar results in a typical fatal case.

TABLE I—RABBIT 1017 LEWISITE, 4 MG/KG ON SKIN

	Hb per cent	Hæma- tocrit	Plasma vol, cc	Corp vol, cc	Total vol, cc	Plasma prot, g per cent	N P N mg per cent
Before lewisite							
3 days before	60	24.6	131	43	174	6.63	40
2 " "	60	22.7	131	39	170	6.66	38
1 day "	59	23.5	140	43	183	6.23	35
0 " "	52	20.3	133	34	167	6.09	44
After lewisite							
1 day after	76	32.3	95	45	140	4.64	46
2 days "	70	30.5	87	38	125	5.03	76
	Died						

The fall in the plasma volume within the first 24 hours is striking. The cell volume remains fairly constant so that there is a considerable fall in the total circulating blood. Provided the animal does not die, the plasma volume remains at a low level for two or three days, and then gradually returns to normal in five to seven days. During the period when the plasma volume is low, the rabbit looks very ill, loses its appetite, has cold ears and extremities and is generally very difficult to bleed from the ear. When the plasma volume begins to return to normal there is usually a great improvement in the general condition of the animal. In fatal cases of lewisite poisoning death usually occurs during this period of shock, but the changes in the gall-bladder and liver might cause death later.

Associated with the fall in plasma volume is a concentration of the blood with a consequent rise in the haemoglobin per cent, red-cell count,

and hæmatocrit These return to normal or fall below normal, when the plasma volume rises

The fall in plasma volume is due mainly to a leakage of plasma from the circulation locally at the site of application of lewisite, and may be in part also in other tissues or the tissues in general remote from the local lesion That the proteins pass out of the circulation is shown by the behaviour of the plasma proteins The total plasma protein concentration falls during the first three days after lewisite application, and then as the blood volume returns to normal the plasma protein level also rises In rabbits the fall in the plasma protein concentration is due in most cases to a fall in the albumin fraction, the globulin remaining constant or rising

The extent of the loss of protein from the circulation can be seen by calculating the total protein in the circulating plasma before and after the application of lewisite The mean results of the experiments on four rabbits shown graphically in fig 1, and the figures for the experiment represented in Table I, are given in Table II

TABLE II—TOTAL CIRCULATING PLASMA PROTEIN IN GRAMS

	Days before lewisite				Days after lewisite						
	3	2	1	0	1	2	3	4	5	6	7
Mean of four rabbits in fig 1			8.1	7.7	4.9	4.5	5.3	6.3			8.2
Rabbit 1017 in Table I	8.7	8.7	8.7	8.1	4.4	4.4	Died				

The fall in the total circulating plasma protein is, therefore, very considerable in lewisite shock If the animal survives, the protein begins to increase on the third day, and by the seventh day is back to normal It is, therefore, fairly quickly replaced

During the period of circulatory collapse with the decreased blood volume and decreased plasma proteins, the blood urea almost invariably rises In some instances this rise is small, in others very great In fig 1 is plotted the N P N of the plasma It rises rapidly from about 30 mg per cent to about 140 mg per cent during the period of shock, but just as rapidly returns to normal when the blood volume returns to its pre-lewisite level This suggests a functional rather than a histological disorder of the kidney A more detailed investigation will be described later

In another group of experiments with rabbits, the blood pressure,



rectal temperature, plasma urea, and hæmoglobin per cent were determined daily before and after skin application of lewisite. In the first series of this group a dose of 4 mg/kg was applied to the skin of five rabbits. The mean results are shown in fig 2. The blood pressure in this series can be seen to fall but slightly with hæmoconcentration. In spite of the decreased blood volume, which generally runs parallel with the hæmoconcentration, the blood pressure has been maintained at a

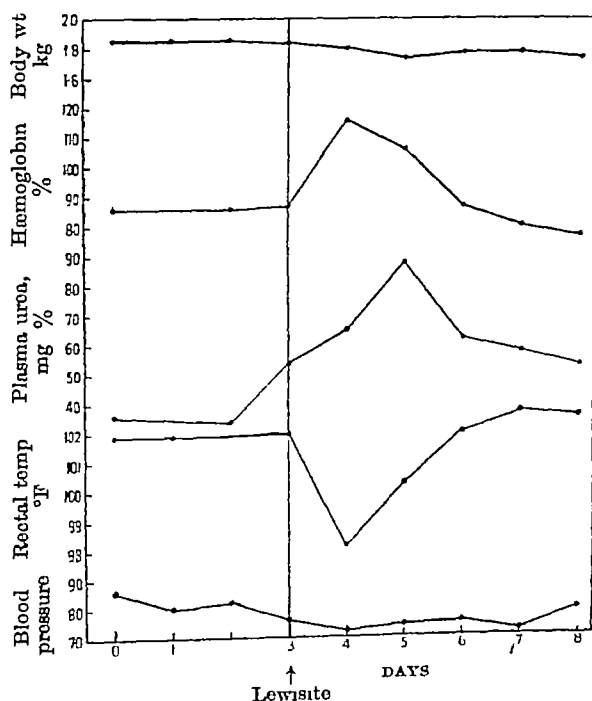


FIG. 2—The effects of skin application of lewisite 4 mg/kg, on blood pressure, rectal temperature, plasma urea, hæmoglobin per cent and body weight in rabbits. Mean of five experiments.

fairly high level by compensatory vasoconstriction. The animal may thus be suffering from considerable plasma loss and fall in blood volume before the blood pressure falls. The blood pressure is, therefore, not a good indication of the fluid loss in the early stages. During the period of hæmoconcentration the rectal temperature fell from 102° F to 98.2° F, and returned to normal as the hæmoglobin per cent returned to normal. In the second series, a larger and lethal dose of lewisite, 6 mg/kg, was applied to the skin of each of four rabbits. The results are shown in Table III. In these rabbits the blood pressure and rectal temperature fell very considerably as the hæmoglobin per cent rose. The fall in blood volume in these cases appears to have been so great that the circulation could not compensate for the loss of fluid by with-

drawal of fluid into the blood stream and by vasoconstriction. A fall in blood pressure is thus an indication of very severe loss of plasma from the blood and is usually a sign of irreversible circulatory failure.

TABLE III —LEWISITE, 6 MG /KG , ON SKIN OF EACH RABBIT

Rabbit	Weight, kg	Hb per cent	Plasma urea, mg per cent	Rectal temp , °F	Blood pressure, mm Hg
1 Before lewisite	2 16	84	53	101 3	82
1 day after	2 04	124	118	94 4	63
	Died				
2 Before lewisite	1 93	91	75	101 7	81
1 day after	1 82	126	98	96 7	70
2 days "	1 76	128	201	93 0	48
	Died				
3 Before lewisite	1 82	94	66	101 6	84
1 day after	1 76	129	113	94 6	52
2 days "	1 70	132	188	93 0	48
3 " "	1 67	120	126	93 0	31
	Died				
4 Before lewisite	1 84	92	58	101 0	80
1 day after	1 76	109	102	96 2	63
2 days "	1 76	112	186	94 0	57
	Died				

resulting in death. In both series it can be seen that the plasma urea rose, the increase being much greater in the second series than in the first.

The blood-pressure changes in acute experiments in dogs are described in subsequent sections.

(b) *Goats* —Goats behave in a manner similar to rabbits following skin application of lewisite, as far as the blood and circulatory changes are concerned. Haemoglobin and red-cell counts have been determined on a large series, but a more complete examination has been made in a small group only. The results are shown in Table IV. In the two goats that died (224 and 255), the plasma volume was reduced by about 50 per cent in each case, while the red-cell volume increased but slightly. The enormous changes in the amount and quality of the circulating blood produced in both these animals a state of profound shock from which they succumbed, despite compensatory vasoconstriction. The blood flow in the peripheral veins appeared to be greatly decreased, as it was much more difficult to get blood from the jugular vein and the blood was much darker than in the same animal before lewisite application. The plasma protein concentration in both these animals fell, but in one the fall was due to a decrease in albumin, and in the other to a fall in the globulin as measured by the sodium sulphate method. However, in all

TABLE IV—EFFECTS OF SKIN APPLICATION OF LEWISITE IN GOATS

	Hb per cent	Hæma tocrit	Plasma vol , c c	Corp vol c c	Total vol , c c	Plas prot	Alb g per cent	Glob , g per cent	N P N mg per cent
<i>Goat 224</i>									
21 11 41	64	22.9	1930	570	2500	8.71	3.74	4.97	30
24 11 41	56	19.8	2090	510	2600	7.59	3.48	4.11	30
Lewisite, 19 hours later	16 mg /kg 125	applied to skin 41.9	980	705	1685	6.34	2.06	4.28	141
<i>Goat 255</i>									
15 12 41	52	17.1	2180	450	2630	6.92	2.33	4.59	36
Lewisite, 20 hours later	10 mg /kg 90	applied to skin 32.4	1225	585	1810	5.30	2.32	2.98	65
<i>Goat 254</i>									
15 12 41	60	23.5	2350	720	3070	7.05	3.01	4.04	40
Lewisite, 16 12 41	10 mg /kg 78	applied to skin 26.0	1885	665	2550	6.28	2.62	3.66	39
17 12 41	72	24.6	2130	690	2820	7.08	2.48	4.60	43
18 12 41	66	22.7	2040	610	2650	6.56	2.41	4.15	39
19 12 41	57	18.7	2700	620	3320	6.29	2.53	3.76	49

three goats the total amount of circulating albumin and of globulin fell considerably as shown in Table V. This indicates that both albumin and globulin have left the circulation.

TABLE V—TOTAL CIRCULATING PLASMA PROTEIN

	Total, g	Albumin, g	Globulin, g
<i>Goat 224</i>			
Before lewisite	159	73	86
19 hours after lewisite	62	20	42
<i>Goat 255</i>			
Before lewisite	151	51	100
20 hours after lewisite	65	28	37
<i>Goat 254</i>			
Before lewisite	165	71	94
1 day after lewisite	118	49	69
2 days " "	151	53	98
3 " " "	134	49	85
4 " " "	160	68	101

The plasma N P N in the first two goats rose considerably during the first 20 hours after lewisite application. The third goat was much less affected than the first two, the plasma volume falling somewhat and subsequently recovering. The general behaviour of the goat after

application of lewisite to the skin is thus similar to that observed in the rabbit

(11) *Intravenous Injection of Lewisite Oxide*—Lewisite causes a loss of plasma from the circulating blood, part at least of which produces the oedema at the site of application. So great is the loss of fluid from the blood, however, that it seems that the local oedema cannot account for all the fluid loss. To see whether the circulating lewisite oxide which results from skin application of lewisite has any effect on the animal, rabbits were given intravenous injections of 1 mg/kg body-weight of lewisite oxide. The results of the changes in hæmoglobin per cent, red-cell count, plasma urea and N P N, and plasma protein concentration are given in fig 3

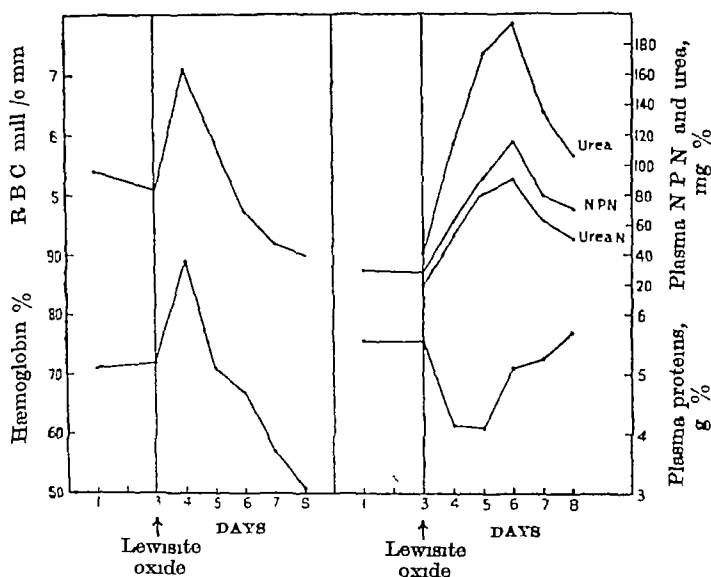


FIG 3—The effects of intravenous injection of lewisite oxide, 1 mg/kg, on hæmoglobin per cent, red cell count, plasma protein per cent, and plasma urea and N P N in the rabbit. Mean of three experiments

A typical shock-like appearance with the blood picture of lewisite shock was evident. In these experiments there was, of course, no local site of injury as after the skin application of lewisite. It appears, therefore, that the lewisite oxide affected the capillaries generally or of those of some organ causing a leakage of fluid from the circulation.

Experiments with the intravenous injection of lewisite oxide in dogs will be described in the sections on lymph drainage and renal function.

These experiments on the blood and circulatory changes so far described, give this picture. The primary factor in the chain of events is the local accumulation of fluid at the site of lewisite application. This leads to a fall in the circulating plasma volume which results in hæmoconcentration and a fall in total blood volume, and a great decrease in

the total circulating plasma protein, both albumin and globulin. The decreased plasma volume causes a withdrawal of non-protein fluid into the circulation through the undamaged capillaries of the body in an endeavour to compensate for the loss of plasma. This causes a fall in the plasma protein concentration. There exists, therefore, a state of hæmoconcentration and hypoproteinæmia with a low plasma protein concentration.

So long as the fall in the plasma volume is not too great, vasoconstriction compensates for the decreased blood volume (and presumably decreased cardiac output) and so maintains the blood pressure at a reasonably high level. When the loss of fluid exceeds a certain limit this vasoconstrictive mechanism fails, and irreversible circulatory failure leads to the death of the animal.

All these events can be explained by a local loss of fluid alone, but there are several factors which suggest that the loss of fluid may not be limited to the local lesion. Firstly, it is known that lewisite oxide is absorbed from the skin after lewisite application, and that lewisite oxide injected intravenously causes a picture of hæmoconcentration and hypoproteinæmia similar to that obtained by the skin application of lewisite. Secondly, the fall in the plasma protein concentration reduces the osmotic pressure in the capillaries in general and so may affect the general fluid exchange, and thirdly, a diminished blood flow to the tissues in general will cause a stagnant anoxæmia which also might affect the fluid exchange throughout the body.

In order to ascertain whether the capillaries in general were affected the lymph drainage of the body was investigated.

## 2 *Lymph Drainage of the Body*

In these experiments, dogs anaesthetized with nembutal have been used. The dogs were in the post-absorptive state, the last meal being given about 18 hours before. No preliminary transfusions were given to increase lymph flow. The lymph ducts necessary for the experiment were cannulated and normal lymph was collected for a period generally of 2 hours. Lewisite was then applied (the amount and site of application will be stated in each experiment) and lymph was collected for varying periods afterwards. The volume and protein content of the lymph were determined. At the same time the blood pressure was determined by recording continuously from a cannula in an artery and blood samples were withdrawn at intervals for the estimations of hæmoglobin per cent and plasma proteins.

In a fairly large series of dogs the normal average protein content of the lymph was as follows: hind leg duct 1.5 per cent, cervical duct 2.6 per cent, right lymph duct 3.6 per cent, and thoracic duct 4.1 per cent, while the plasma protein concentration was on the average 5.5 per

cent In all the experiments with lewisite the lymph has been collected mainly from the hind-leg duct, the cervical duct, and the right lymph duct Some experiments were performed using the thoracic duct, but the results obtained with this duct are difficult to interpret The lymphatics draining into the thoracic duct come mainly from the abdominal viscera and the flow is very large This large lymph flow compared with the flow from the other main lymph ducts is due to the special function of absorbing chyle If ascitic fluids are introduced into the peritoneal cavity, practically no change is observed in the thoracic duct flow [Watkins and Fulton, 1938] Thus, any increase from this cause, *e g* in peritoneal effusion, is masked by the large flow from the gut The protein content of thoracic duct lymph is also very high and approaches that of plasma A peritoneal effusion might, therefore, be caused with an increase in lymph flow and an increase in protein content, but these changes could be masked by the large flow of lymph rich in protein from the viscera

The effects of lewisite on the lymph drainage in dogs have been investigated in three sections

- (i) The lymph draining the area of skin contamination
- (ii) The lymph draining tissues other than at the site of contamination
- (iii) Lymph drainage of the body in general after the intravenous injection of lewisite oxide in which case there is no local area of contamination

(i) *Lymph Drainage of the Area Contaminated with Liquid Lewisite* —

In this series of experiments, one of the cervical ducts and one of the ducts of the hind leg were cannulated in the same dog In this way normal lymph, coming from the head and neck, especially the nose and pharynx, and from the lower part of the hind leg, was collected for about 2 hours A small, non-lethal dose of lewisite, 2 mg /kg body-weight, was then applied to the mucosa of the nose and to the skin of the hind foot Lymph was then collected for several hours The lewisite caused oedema of the nose with free fluid in the nasal cavities and oedema of the foot The first visual signs of oedema appeared about half an hour after the application

The results of experiments in three dogs were almost identical, and a typical case is shown in fig 4 The lymph flow from both the cervical duct and the leg duct increased considerably The mean flow from the cervical duct in the three dogs was 2.8 c.c./hr before the application of lewisite This flow increased to a maximum of 9.5 c.c./hr The maximum flow generally occurred 2 to 3 hours after application of lewisite and then decreased slightly In the leg duct the mean flow of the three dogs before application of lewisite was 2.0 c.c./hr This increased to a maximum of 8.8 c.c./hr after lewisite The increased

flow of lymph from the local area of contamination is thus on the average about four times the normal flow in the stage of acute oedema formation

The changes in the protein content of the lymph were as dramatic as the changes in the lymph flow. In the three dogs the mean protein concentration of the cervical lymph was 2.2 per cent before lewisite contamination. This rose to a mean maximum of 4.7 per cent after lewisite. Likewise the mean protein concentration of the leg lymph was 1.7 per cent before lewisite and rose to a mean maximum of 4.9 per cent after lewisite. The actual rise in the protein concentration of the lymph can best be seen in fig 4, where the plasma proteins are also

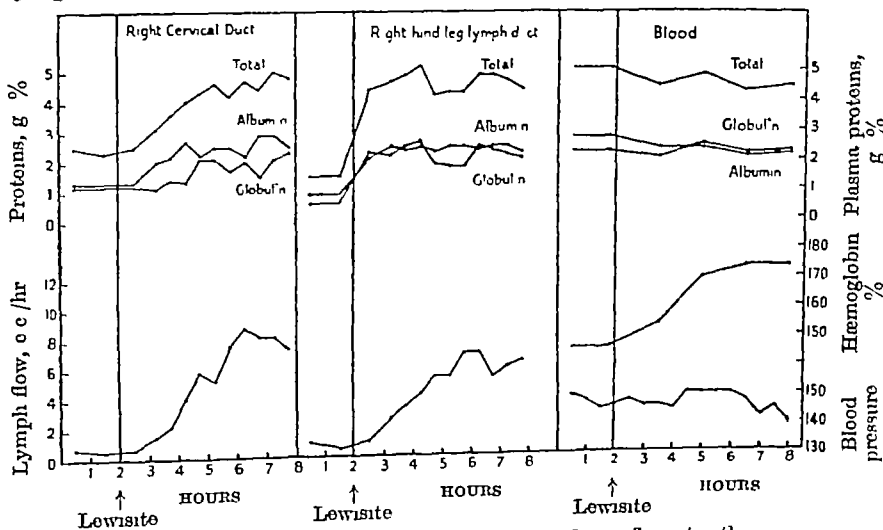


FIG 4—The effects of application of lewisite, 2 mg/kg, to the mucous membrane of the nose and to the skin of the right hind foot on the lymph from the right cervical duct and right hind leg lymph duct in the dog under nembutal anaesthesia

plotted. In all cases both in the cervical and in the leg lymph, the protein concentration rose rapidly and soon reached a fairly steady level which was approximately the same as the protein content of the plasma. The albumin, globulin, and total proteins in the lymph after lewisite were all very similar to the concentrations in the plasma.

During the 6 hours after the application of lewisite, the loss of fluid from the blood caused a rise in the haemoglobin per cent, but was not severe enough to cause any appreciable change in the blood pressure.

These experiments show clearly that the capillaries are rendered completely permeable to the plasma proteins by the action of lewisite. The protein concentration in the tissue fluid thus becomes the same as the protein concentration of the plasma, with a resultant upset in the osmotic balance between the blood and tissue fluid through the capillary membrane. The proteins of the tissue fluid can only be drained away

by the lymph, so the amount of œdema will depend to a large extent on the lymph flow

(n) *Lymph Drainage of the Body other than the Contaminated Area* — In these experiments a lethal dose of lewisite, 15 or 20 mg/kg, was applied to the clipped skin of the left foreleg. A large area of local œdema rapidly developed. The ducts cannulated were the cervical, hind leg, and right lymph duct. All of these ducts were draining parts of the body remote from the contamination.

The results of a typical experiment are shown in fig 5. The effects on the flow of the right lymph duct and left cervical duct are depicted

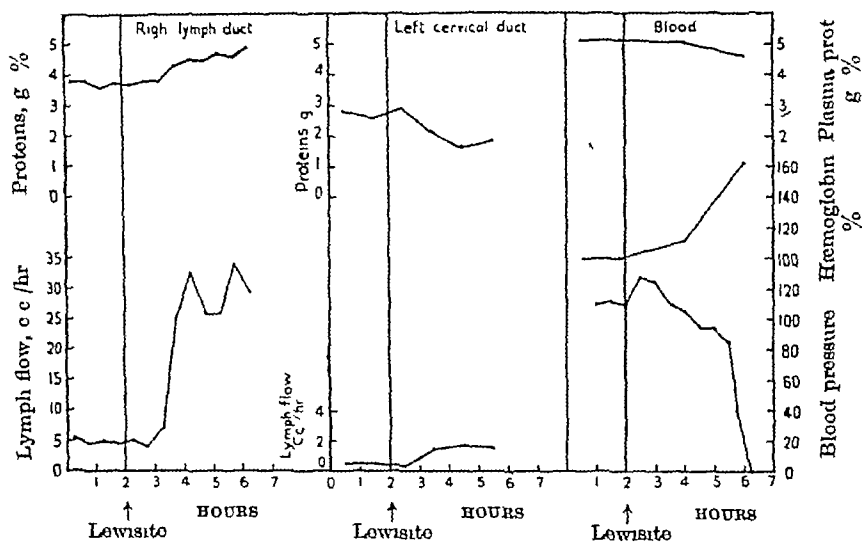


Fig 5 — The effects of skin application of lewisite, 15 mg/kg, to the left foreleg, on the lymph from the right lymph duct and the left cervical duct in the dog, under nembutal anaesthesia

The effects on the lung of skin application of lewisite vary somewhat in different species. In the rabbit, pulmonary œdema after lewisite on the skin is not as common as in the dog and goat. When pulmonary œdema develops as a result of skin application of lewisite, the lymph flow of the right lymph duct which drains the lungs is very greatly increased. The dog in fig 5 had a normal right lymph duct flow of about 5 c.c./hr. This increased to 30–35 c.c./hr 3 to 4 hours after lewisite application. The lymphatics were thus draining a considerable amount of fluid from the lungs, but in spite of this the dog died 4½ hours after the application of lewisite with massive pulmonary œdema. The rate of production of tissue fluid in the lung thus greatly exceeded the removal by the lymphatics. The protein concentration of the lymph was 3.7 per cent before lewisite and rose to 4.9 per cent after lewisite. As can be seen from fig 5, this closely resembled the protein concentration of the plasma.



The effect of the lewisite on the lymph from the cervical duct was different from that on the right lymph duct. There was a slight increase in lymph flow, but associated with this increased flow was a decrease in the protein concentration. Similar results were obtained in experiments on two other dogs. The increased flow in this case is probably not due to an increased permeability of the capillaries to protein, as is the case with the lung lymph changes. The significance of these changes will be discussed later.

The hæmoglobin per cent rose from 100 to 164 in the 4 hours after lewisite, showing very rapid and considerable hæmoconcentration. The blood pressure was maintained in the early stages, but then fell, slowly at first, but towards the end very steeply. Thus, as the plasma volume decreased, the vasoconstrictive mechanism adequately compensated for decreased cardiac output for a while, but then rapidly failed.

In other similar experiments, the lymph from the hind leg duct did not change appreciably from normal, either in flow or protein concentration. It is, therefore, presumed that the permeability of the capillaries in this region was not altered.

(iii) *Lymph Drainage of the Body after Intravenous Injection of Lewisite Oxide*—In these experiments the cervical, hind leg, thoracic and right lymph ducts have been cannulated. Lewisite oxide, in doses of 1 or 1.5 mg/kg body-weight, was injected intravenously. A typical result is depicted in fig. 6.

This represents the effects on the right lymph duct, cervical duct, and hind leg duct. In this dog pulmonary oedema was very considerable, and associated with this pulmonary oedema was an increase in the lymph flow from about 1 c.c./hr to 8 c.c./hr. In the last hour when the animal was becoming moribund, the flow decreased. The protein concentration of the lymph rose from 3.3 per cent to 5 per cent, so that it became identical with that of the blood plasma. The flow from the cervical duct increased slightly, but the protein concentration decreased. The lymph from the leg duct showed practically no change, either in flow or protein content.

In three other experiments results similar to those shown in fig. 6 were obtained. In one such experiment the lymph flow from the right lymph duct increased from 8 c.c./hr to 76 c.c./hr.

In two experiments lewisite oxide had no significant effect on the flow or protein concentration of the thoracic duct lymph.

The blood changes in all these experiments are typical of lewisite shock. The hæmoglobin per cent rises steeply, the blood pressure is maintained for a while by compensatory vasoconstriction, but then falls steeply until death.

In this series of dogs the most prominent lesion after the intravenous injection of lewisite oxide was pulmonary oedema. As the oedema

rapidly developed, the lymphatics draining the lungs carried away a greatly increased amount of lymph which resembled blood plasma in its composition. Although the lymph flow may be increased tenfold, the actual amount of fluid drained is generally small compared with the amount of excess tissue fluid produced in the lungs.

The mode of production of the oedema fluid of the lungs can be explained by an increased permeability of the capillaries of the lungs to protein as in the case of the skin capillaries after lewisite application

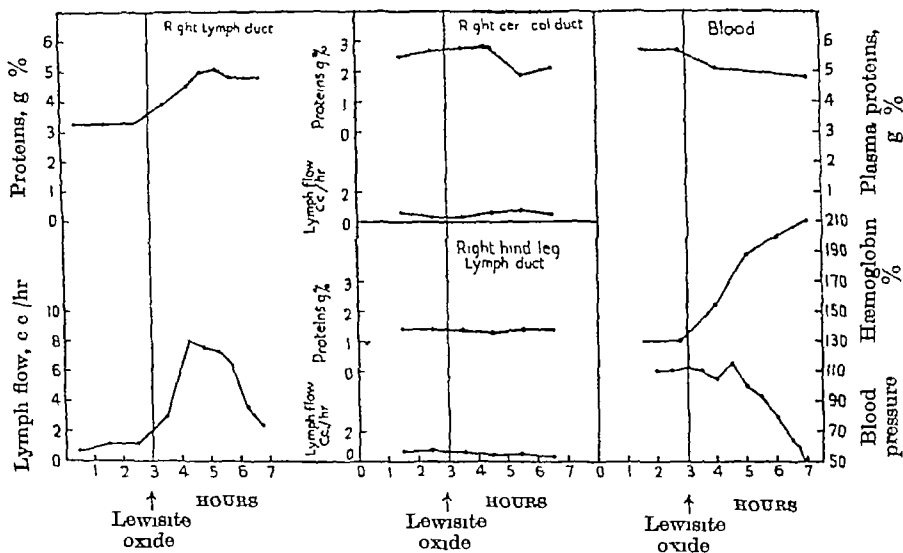


FIG. 8.—The effects of intravenous injection of lewisite oxide, 1 mg/kg, on the lymph from the right lymph duct, right cervical duct, and right hind leg duct in the dog, under nembutal anaesthesia

to the skin. The effects of lewisite oxide on the capillaries of other parts of the body are not the same as on those of the lungs. The reasons why the lung capillaries are affected may be because the lewisite oxide, when injected intravenously or when absorbed after skin application of lewisite, passes through the lung capillaries before any others. Bruner [unpublished] has shown that intra-arterial injection of lewisite oxide, 1 mg/kg, causes marked oedema of the area supplied by that artery in rabbits and dogs, but in dogs pulmonary oedema develops as after intravenous injection. It thus appears that all capillaries of the body will be affected by lewisite oxide if the concentration is great enough, but the pulmonary capillaries are more sensitive in dogs. When minimal lethal doses of lewisite are applied to the skin or of lewisite oxide injected intravenously, the concentration of lewisite oxide in the general systemic circulation is not sufficient to affect the permeability of the capillaries in general. It, therefore, appears that the circulatory changes in lewisite poisoning are due mainly to loss of fluid locally

and in the lungs, the effect on the lungs varying in different species of animal

### 3 The Effect on Renal Function

(1) *Chronic Experiments in Unanaesthetized Rabbits*—It has already been seen that the plasma N P N and urea rise rapidly during the period of hæmoconcentration and fall as rapidly during the recovery of the blood volume to normal. This rise in plasma N P N suggests a failure of renal function. Histologically there is generally little change in the kidney, so the disorder seems a functional one associated with the circulatory disturbance of shock. The effects of the skin application of lewisite, 4 or 5 mg/kg, on the plasma N P N and urea in a number of rabbits are shown in Table VI. It can be seen from this table that the changes are variable, but that in practically every case there is a rise in the N P N and urea.

TABLE VI—THE PLASMA N P N AND UREA AFTER SKIN APPLICATION OF LEWISITE

	Days before lewisite		Days after lewisite					
	2	0	1	2	3	4	5	6
1 N.P.N	43	40	57	188	Died			
Urea	55	52	94	267				
2 N P N	49	49	41	53	56			46
Urea		61	73	85	75			59
3 N P N	41	30	32	40	46	40	34	
Urea		44	38	50		61	36	
4 N P N	20	29	68	59	48	49	47	
Urea		42	99		63	61	45	
5 N P N	34	31	57	78	148	262	191	
Urea		44	90	145	263	383	311	
6 N P N	34	31	34	48	51	42	40	
Urea	39	30	50	75	64	49	42	
7 N P N	20	26	50	103	193	Died		
Urea			81	193	480			
8 N P N	32	24	94	164	202	250	302	
Urea		36	176	285	398	409	533	
9 N P N	44	37	52	168	177	73		45
Urea	49	43	74	300	285	138		47
10 N P N	34	32	42	42	50	46		34
Urea	43	42	35	74	56	50		46
11 N P N	36	35	39	35	49	38		20
Urea	41	47	31	63	70	53		32
12 N P N	34	41	65	106	71	58		
Urea	42	48	93	133	86	72		
13 N P N	32	36	70	125	80	59		
Urea	38	55	92	176	111	85		
14 N P N	31	30	48	50	38	38		
Urea	38	53	67	61	44	39		

In order to ascertain the cause of this rise in blood urea, experiments were carried out in which the blood urea and the daily output of urea in the urine were determined before and after the skin application of lewisite. Male rabbits were used as the experimental animal. They were kept in metabolism cages and catheterized each morning to obtain the daily output of urine. The amount of food given to each rabbit daily was approximately constant, but the protein content was not measured. The exogenous protein metabolism would therefore vary somewhat.

Of a series of nine rabbits, typical results are shown in figs 7 and 8

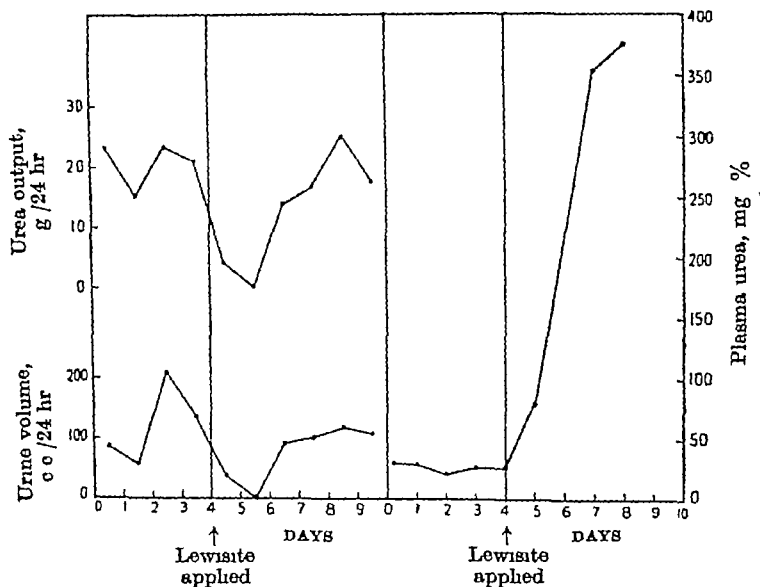


FIG 7—The effects of skin application of lewisite 4 mg/kg, on the urine volume, urea output and plasma urea in the rabbit

In the experiment depicted in fig 7, the urine output decreased on the first day after lewisite and there was a complete suppression of urine on the second day. The urine output then increased, but in spite of this the plasma urea continued to rise rapidly until the death of the animal. The total urea excretion during the first two days after lewisite was almost nil, as very little urine was being excreted. The retention of urine in this case is, therefore, sufficient to raise the blood urea to about 400 mg per cent, provided the protein breakdown continues at the same rate as before lewisite. This animal was very ill, and the cause of the suppression of urine was probably a considerable fall in the blood pressure at the height of the shock. This type of reaction is seen only in the severe and fatal cases. In the less severe cases that suffer from considerable haemoconcentration for two or three days but then recover rapidly, complete suppression of urine is not observed. A typical

case is represented in fig 8 During the period of shock after lewisite, the daily output of urine falls The urine, however, contains a very high concentration of urea, sometimes as high as 6 per cent, so that the daily urea output actually increases In spite of this, the plasma urea also rises The daily urea output is often doubled, from about 2 g /day to 4 g /day, the greatest rise being on the third and fourth days after lewisite application, when the animal begins to recover from the shock and the plasma urea begins to fall

The increased urea output during the first week after lewisite

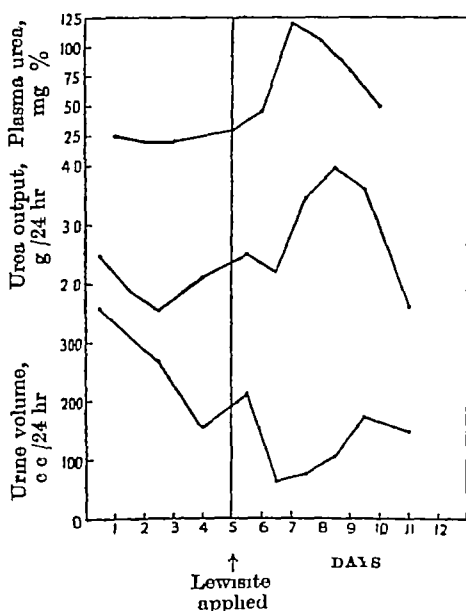


FIG 8—The effects of skin application of lewisite, 4 mg/kg, on the urine volume, urea output, and plasma urea in the rabbit

application, as represented by the hump of the curve in fig 8, means a greatly increased protein breakdown This increased protein katabolism cannot be caused by an increased protein intake in the diet, because the rabbits ate less during the period of shock than normally Thus, during the period of haemoconcentration after lewisite, the protein breakdown is increased, but the kidneys are not able to excrete all the urea formed, although they excrete a urine which contains up to 6 per cent urea Part of the excess urea formed is thus dammed back in the blood and tissues, causing a rise in blood urea When the plasma volume begins to return to normal on the third and fourth days after lewisite, the urine output increases and with this increased flow of urine the excess urea in the tissues is gradually excreted The plasma urea concentration then falls to normal limits Since the stimulus to excess tissue breakdown is removed when the plasma volume recovers,

the protein katabolism and therefore the urea output soon fall within the normal range

Thus in lewisite shock is seen the typical syndrome associated with dehydration, viz oliguria, increased protein katabolism with increased urea output, and a rise in blood urea. The fact that the kidney can concentrate urea to such an extent confirms the lack of histological damage and suggests that the nitrogen disturbance is mainly a functional one, probably a decreased circulation through the kidneys

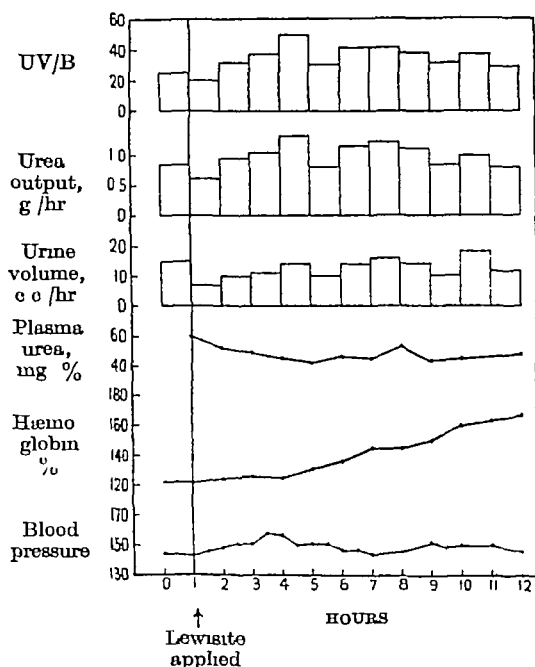


FIG 9—The effects of skin application of lewisite, 7 mg/kg, on the blood pressure, haemoglobin per cent, plasma urea, urine volume and urea output in a dog, under sodium barbitone anaesthesia

(ii) *Acute Experiments in Anaesthetized Dogs*—The changes in renal function and protein metabolism are best seen in the chronic experiments just described, for the changes involved are gradual in onset and take some time to develop. The complete syndrome described in the previous section can, therefore, be seen only during an interval of a few days or a week. Acute experiments with anaesthetized dogs have been performed only to investigate the very early stages, and especially the effects of a relatively large dose of lewisite which results in death in a few hours.

Investigations were, therefore, made with dogs anaesthetized with sodium barbitone (1 c.c. of a 10 per cent solution per pound body-weight, intravenously). Male dogs were used. A catheter was passed into the bladder and hourly samples of urine were collected. Hourly samples of

blood were withdrawn from the jugular vein for the estimation of the hæmoglobin per cent and plasma urea. The blood pressure was recorded continuously from the carotid artery.

The effects of a non-lethal dose of lewisite are shown in fig 9. This experiment was followed for 11 hours after the skin application of 7 mg lewisite/kg body-weight. During this time the blood pressure remained fairly constant, and the urine volume, urea output, and urea clearance also remained constant in spite of a gradual hæmoconcentration. The urea clearance was calculated from the ratio  $UV/B$ , where  $U$  = urine urea concentration in mg/c.c.,  $V$  = volume of urine in c.c./min., and  $B$  = plasma urea concentration in mg/c.c. In acute experiments of

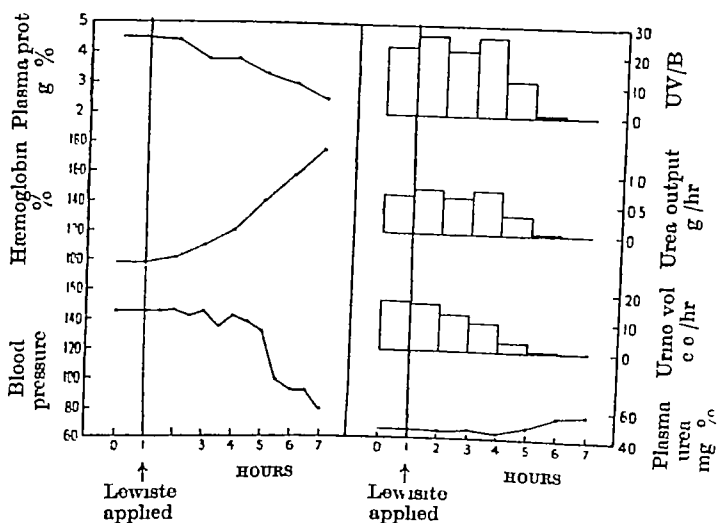


FIG 10—The effects of skin application of lewisite 25 mg/kg, on the blood pressure, hæmoglobin per cent, plasma protein per cent, plasma urea, urine volume and urea output in a dog, under sodium barbitone anaesthesia.

this nature the time is too short to demonstrate the oliguria, increased blood urea and increased urea output as seen in the chronic experiments.

With larger doses of lewisite on the skin, or with lethal doses of lewisite oxide intravenously, the urine ceases to form when the blood pressure falls considerably. A typical experiment is shown in fig 10. It shows the production of anuria in the severe cases of lewisite shock with a considerable fall in blood pressure.

In all these cases histological examination of the kidney at the end of the experiment showed little damage to the glomeruli or tubules.

All these experiments support the view already stated that the effects on renal function are secondary to the circulatory changes, and are not due to histological damage of the renal cells. With a sudden large outpouring of fluid resulting in a fall in blood pressure, anuria may follow if the fall in blood pressure is sufficiently great to prevent filtration.

In the less severe cases the kidney is not able to deal efficiently with the excess urea formation caused by the increased protein catabolism, because of the decreased blood flow through the kidney resulting from the vasoconstriction which compensates for the decreased cardiac output during the state of shock

#### 4 *Hæmolytic Anæmia following Lewisite Poisoning*

The sudden fall in plasma volume with resultant hæmoconcentration is the outstanding feature during the first few days after skin application of lewisite. Following this hæmoconcentration, the hæmoglobin per cent and red-cell count generally fall considerably below the normal level (*cf* fig 1). From the hæmoglobin per cent and the total blood volume the actual amount of hæmoglobin in circulation can be calculated. These calculations have been made in the four individual experiments on rabbits, the mean results of which are represented in fig 1. The actual amounts of hæmoglobin in the circulating blood before and after lewisite application are given in Table VII. These figures show that following the hæmoconcentration the hæmoglobin per cent and red-cell count indicate a true anæmia.

TABLE VII—THE TOTAL HÆMOGLOBIN IN GRAMS IN THE BLOOD OF FOUR RABBITS BEFORE AND AFTER SKIN APPLICATION OF LEWISITE

	Days before lewisite		Days after lewisite					
	2	0	1	2	3	4	7	9
1	20.6	20.4	19.0	16.4	16.5	13.9	15.1	16.2
2	20.2	19.1	19.1	17.4	18.8	19.1	15.8	14.6
3	19.9	18.8	19.6	20.4	18.9	14.0	16.5	14.9
4	24.3	22.2	21.6	22.6	21.3	18.4	13.8	14.3
Mean	21.2	20.1	19.8	19.2	18.9	16.4	15.3	15.0

This anæmia was investigated further. Rabbits were given skin applications of lewisite in doses of 4 mg/kg body-weight. Blood changes were followed at daily intervals for three weeks. The bone marrow was examined by means of sections stained by Turnbull's Jenner method, animals being killed at intervals after lewisite application for this purpose. One group of animals received intramuscular injections of iron ( $1/8$  to  $1/3$  c.c./kg of Inject. Ferri B.P.) for fourteen days after lewisite contamination. Blood examination included enumeration of red cells and reticulocytes, hæmoglobin per cent, size of red-blood cells and study of blood smears by Jenner's method.



In fig 11 are shown the hæmoglobin per cent, red-cell count and reticulocyte count in a group of three rabbits before and following a non-lethal dose of lewisite. It can be seen that after the hæmoconcentration there is a gradual fall in the hæmoglobin per cent and red-cell count. These values begin to increase again ten to fourteen days after the lewisite. The reticulocytes begin to increase soon after the red cells decrease and reach a peak three days after the lowest values for red cells and hæmoglobin are reached. Abnormal red cells appear in the blood

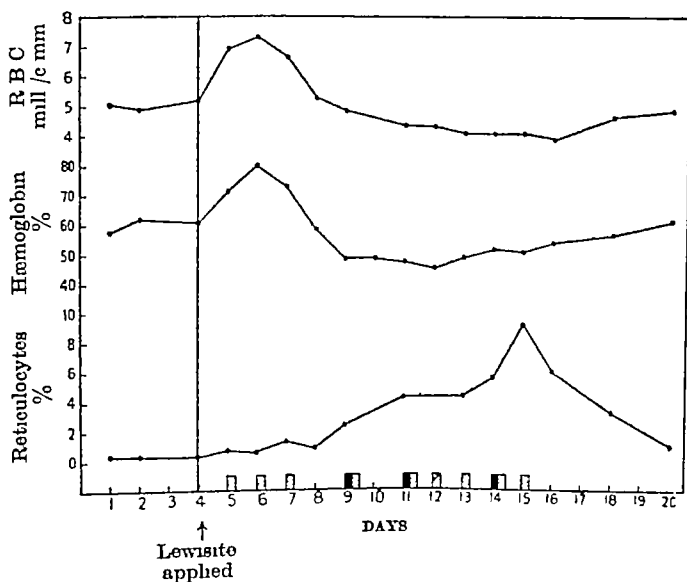


FIG 11—The effects of skin application of lewisite 4 mg/kg, on the hæmoglobin per cent, red cell count and reticulocyte count in rabbits. Mean of three experiments

■ = polychromasia    stippled cells  
 ▨ = normoblasts

stream about the time of the most marked reticulocyte response. Measurement of the diameters of the red cells shows that there is no change in size.

It appears, therefore, that there is a true anæmia with a typical compensatory bone marrow response which is responsible for replacement of lost red cells. It is probable that the anæmia is hæmolytic in character and not due to a disturbance of the bone marrow, for histological examination of the marrow at various stages after lewisite fails to show degenerative changes in the marrow cells, the reticulocyte response indicates that the marrow is quite capable of producing new red cells, and abnormalities in the size of the red cells are not seen.

The administration of iron did not influence the course of the anæmia. This is not surprising since in all probability there is no iron deficiency.

The iron from the destruction of the red cells is probably used again in the production of further cells

### *Other Blood Investigations*

*Sedimentation Rate*—The sedimentation rate as determined with Westergren tubes has been carried out on a series of rabbits before and after skin contamination with lewisite, 4 mg/kg body-weight. The results are somewhat variable. In one series of six animals there was little increase in the sedimentation rate following lewisite, while in a second series of four animals the increase was appreciable corresponding with the period of anæmia.

*Corpuscular Fragility*—In view of the anæmia that develops after the period of hæmoconcentration, the fragility of the corpuscles was determined in a series of rabbits before and after skin contamination with liquid lewisite. No significant changes were observed.

*Coagulation Time*—Investigations in rabbits showed that this did not change following lewisite contamination.

## DISCUSSION

### *Site of Action of Lewisite*

(a) *Local*—When liquid lewisite is applied to the skin of animals, a central area of necrosis surrounded by an area of erythema rapidly develops. In the skin and the subcutaneous region surrounding the site of application, excess tissue fluid accumulates. The tissues, including the capillaries, in the central area of the lesion are killed, but surrounding this area, the capillaries are affected in such a way as to allow a rapid increase in the formation of tissue fluid. There is probably a gradation in the damage done to the tissues, descending in degree from the centre towards the periphery of the lesion, beyond which the capillaries are normal. It is this damage to the capillary membrane that is of such importance in the sequence of events that follow skin contamination with lewisite.

The degree of damage to the capillary membrane can be seen in the experiments on the lymph drainage of the contaminated area. The protein content of the lymph coming from this region soon reaches the level in the blood plasma. The capillary permeability is, therefore, increased so as to allow the free passage of the plasma proteins, the concentration of both albumin and globulin in the lymph rising to the same level as in the plasma. This indicates that the capillary filtrate is identical with plasma, since, the concentration of protein in the tissue fluid and plasma being equal, there can be little or no reabsorption of fluid into the local capillaries. The only drainage of the œdema fluid

will, therefore, be by way of the lymphatics, and although the lymph flow increases greatly, the production of œdema fluid proceeds at a much greater rate than the removal

(b) *General* —Some of the lewisite applied to the skin is absorbed into the blood stream as lewisite oxide. It has also been seen that lewisite oxide injected intravenously into animals will also cause a loss of fluid from the blood. It therefore appears that in lewisite contamination of the skin, the local œdema is not the only loss of fluid from the blood stream.

The problem of this general loss of fluid was examined by a study of the gross pathology and of the lymph drainage of the body. Gross œdema other than at the site of application varies somewhat in different species of animal and also in individual animals of the same species. In the dog and goat pulmonary œdema and pleural effusion are more marked than in the rabbit. Peritoneal effusions sometimes occur also, but are not so great as the pulmonary œdema often observed in the dog. Many rabbits showed no macroscopic signs of pulmonary œdema, and even after very large and repeated plasma transfusions the lungs of rabbits appeared normal with a normal lung/heart ratio. The muscle and skin which form the bulk of the soft tissues of the body do not appear to contain an increased amount of tissue fluid.

The investigation of the lymph drainage of various regions of the body was undertaken to ascertain the state of the capillaries in general. The results of these experiments indicated that the only capillaries with an increased permeability were those of the lungs. The damage to the lung capillaries was such as to render them freely permeable to the proteins so that pulmonary œdema ensued. The lung capillaries are probably more easily damaged because the absorbed lewisite oxide first passes through the lungs, but this is not the entire explanation since Bruner showed that in dogs after intra-arterial injection of lewisite oxide, pulmonary œdema often develops as well as œdema in the region supplied by that artery. It appears, therefore, that the lung capillaries are more easily damaged by lewisite oxide than capillaries in the rest of the body.

#### *Effects of Fluid Loss on Blood and Circulation*

The loss of fluid from the circulation has been seen to produce shock with signs and symptoms similar to those produced by thermal burns. The primary change is the fall in plasma volume. As this progresses compensatory vasoconstriction maintains the blood pressure for a time, but as the plasma volume falls still further this compensatory mechanism fails and the blood pressure falls. In those animals in which the blood pressure falls considerably, death almost invariably results. Thus, as in other types of shock, the hemoconcentration is a much better guide to early fluid loss than is the blood pressure. Another good indication of

the degree of shock in rabbits is the body temperature which falls as the hæmoglobin per cent rises

The experiments described indicate that lewisite shock is fundamentally similar to shock produced by thermal burns although in the latter the loss of fluid is probably local only and the onset of œdema is much more sudden than with lewisite. The blood and circulatory changes in both cases are, however, similar, so no detailed discussion of these phenomena in lewisite shock is necessary

### *Effects on Renal Function and Nitrogen Metabolism*

In lewisite poisoning the loss of fluid from the blood results in an oligæmia, and the animals in all cases become very thirsty. If the fall in plasma volume is great enough to cause a substantial fall in blood pressure, urinary secretion ceases. The effective pressure in maintaining filtration in the renal glomeruli is the balance between the capillary pressure and the osmotic pressure of the plasma proteins. With a fall in arterial pressure it does not follow that the capillary pressure must also fall, but it seems probable that with a large fall in arterial pressure, the capillary pressure will also fall. When this pressure is exactly balanced by the osmotic pressure of the proteins in the plasma, urine will not be formed. In most cases of lewisite poisoning slight albuminuria occurs but the concentration of albumin in the urine is very small compared with that in the plasma. Therefore the concentration of albumin in the glomerular filtrate will not be sufficient to alter to any appreciable extent the effective osmotic pressure of the plasma proteins. It has been seen in acute experiments in dogs that urine secretion ceases with a considerable fall in blood pressure. In these cases death usually follows rapidly.

The changes in the less severe, non-lethal cases are of more interest. During hæmoconcentration the urinary volume falls, but the urea concentration in the urine increases to such an extent that the daily output of urea is increased. The plasma urea increases at the same time. As soon as the anhydremia is overcome and the plasma volume restored to normal, the disturbance in nitrogen metabolism ceases.

Similar changes have been observed in excessive water loss from many causes, such as vomiting as in intestinal obstruction and diarrhœa as in dysentery or cholera [cf Marriott, 1923]. Mackay and Mackay [1924] have combined complete starvation with intravenous injections of sucrose to produce rapid anhydremia. This led to an increased urea excretion in the urine and a rise in blood urea. They ascribe this increased protein metabolism to an increased tissue catabolism resulting from dehydration. Bywaters and Popjak [1942] have also described an increased tissue breakdown with increased urea formation in con-

ditions of shock produced by compression and release of the thigh in rabbits

In all these conditions, fluid loss from the blood is a common factor. It therefore seems that the increased protein catabolism can be explained by the anhydræmia. The breakdown of muscle occurs rather than fat, because more water is liberated. Calorie for calorie the intrinsic water liberated by the combustion of protein and fat is about the same, but the extrinsic water liberated is very much greater in the case of muscle tissue, which contains 75 per cent water in the rabbit, than in the case of fat.

This increased urea formation throws an extra burden on the kidney at a time when this organ is trying to conserve water. The kidney secretes a highly concentrated urine with a urea percentage up to 6 per cent, in an endeavour to retain water and at the same time get rid of excess urea. This suggests that the power of the tubules to concentrate the glomerular filtrate is not inhibited. In spite of this increased urea output the plasma urea rises, which indicates that the filtration of plasma by the glomeruli cannot be increased sufficiently, probably because of a decreased blood flow through the kidney due to vasoconstriction.

### *Secondary Anæmia*

It has been seen that after lewisite shock there is an actual decrease in the amount of circulating hæmoglobin. This anæmia is most marked about seven days after lewisite application and is followed by a reticulocyte response and a subsequent return to normal. The cause of the anæmia appears to be a destruction of some of the circulating red cells as the bone marrow shows no histological change.

It may be that the circulating lewisite oxide is the destructive agent, but it seems more probable that the anæmia is non-specific resulting from the anhydræmia. Marriott [1923] in a review of anhydræmia states that in this condition red-blood cells are destroyed and anæmia is evident when the blood volume is restored to normal. In thermal burns secondary anæmia often results, but the cause is at present controversial [cf Harkins, 1942]. Since, in all these cases of anhydræmia, anæmia is often seen, it is probable that the secondary anæmia of lewisite poisoning is not due to the specific action of lewisite, but to the anhydræmia itself.

### SUMMARY

The effects on the water balance of the body when lewisite is applied to the skin of animals have been investigated using rabbits, goats, and dogs.

Skin application of liquid lewisite causes vesication in man, and in animals a central area of necrosis surrounded by an area of subcutaneous œdema. Some of the lewisite is absorbed into the blood stream and circulates as lewisite oxide.

Experiments on the composition and flow of lymph in dogs show that the skin capillaries at the site of application of lewisite are rendered freely permeable to the plasma proteins. Local œdema, therefore, results because the osmotic effect of the plasma proteins is neutralized by the same concentration of proteins in the tissue fluid.

In dogs the lung capillaries are also often rendered freely permeable to the plasma proteins, after a lethal dose of lewisite on the skin, with resultant pulmonary œdema and a great increase in lymph flow from the right lymph duct. Elsewhere in the body the study of the lymph does not show any increased permeability of the capillaries.

The effect of the fluid loss from the circulation is primarily a fall in plasma volume. This causes hæmoconcentration, as is evident from a rise in the hæmoglobin per cent, red-cell count, and hæmatocrit, a fall in the plasma protein concentration with hypoproteinæmia, a fall in rectal temperature, and if the fluid loss is great enough, a fall in blood pressure. This picture of shock is similar to that seen in thermal burns.

Associated with these circulatory changes are changes in renal function and nitrogen metabolism. With a substantial fall in blood pressure, urine secretion ceases. In less severe cases the syndrome associated with anhydræmia is observed, viz oliguria, increased daily urea output and increased plasma urea. This means an increased endogenous nitrogen metabolism with breakdown of muscle tissue, which liberates more water than would the oxidation of fat.

The period of hæmoconcentration is followed by one of anæmia, the actual amount of circulating hæmoglobin falling considerably. This anæmia is at its peak about seven to ten days after lewisite application and is probably due to destruction of red cells as a result of the anhydræmia. It is followed by a reticulocyte response and recovery to normal in about two weeks. The administration of iron does not affect the course of the anæmia.

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ATROPHY OF THE SUPRAOPTIC AND PRAVENTRICULAR  
NUCLEI AFTER INTERRUPTION OF THE PITUITARY  
STALK IN DOGS By W J O'CONNOR From the  
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FISHER, INGRAM, and RANSON [1935] found that in cats most of the cells of the supraoptic nuclei had entirely disappeared some months after removal of the posterior lobe or section of the supraoptico-hypophyseal tracts, and later Magoun and Ranson [1939] in the monkey and Rasmussen [1939] in the dog and rat counted the cells in the supraoptic nuclei of normal animals and of animals after section of the pituitary stalk, each finding after operation less than 20 per cent of the normal number of cells

The characteristic cells of the paraventricular nuclei resemble those of the supraoptic nuclei, but cells of different types are intermingled and the margins of the nucleus are not well defined, so that its atrophy is difficult to establish. Fisher, Ingram, and Ranson [1935], Ingram and Fisher [1936], Magoun and Ranson [1939], Heinbecker and White [1941], and Rasmussen [1939] have thought that they could detect slight or moderate atrophy of parts of the paraventricular nuclei after section of the pituitary stalk, but cell counts by Pickford and Ritchie [1945] indicated much atrophy. In this paper counts are reported of the cells of the supraoptic and paraventricular nuclei of normal dogs and of dogs submitted to operative procedures to interrupt the hypophyseal tracts, atrophy occurred in both supraoptic and paraventricular nuclei.

Heinbecker and White [1941] submitted dogs to various operative procedures in the region of the hypophysis and hypothalamus, estimated the number of cells remaining in the supraoptic nuclei from an examination of a few typical sections, and claimed a correlation between the degree of diabetes insipidus and the extent of the atrophy of the nuclei—the fewer the residual cells, the greater the polyuria. The data presented in this paper do not confirm the claim of Heinbecker and White.

#### MATERIAL AND METHODS

*Material*—Cell counts were made in the following operated bitches—

(1) Four animals in which the supraoptico-hypophyseal tracts had been cut by the operation described in a previous paper [O'Connor,



1946], where a full description is given of the extent and results of the lesion in these animals

(2) Five animals from which the posterior lobe had been removed leaving the pituitary stalk, as described by O'Connor and Verney [1942]

(3) Four animals in which an attempt was made at a second operation to section or destroy the median eminence after the posterior lobe had already been removed, one animal in which extensive destruction of the median eminence resulted from the attempt to remove the posterior lobe, and one in which removal of the posterior lobe was incomplete. Where reference is made to these animals the post-mortem findings will be specifically given

Counts were made in control bitches of the same weight range as the operated animals

*Method of Cell Counting* —The animals were killed by bleeding from one carotid under chloralose or chloroform and ether anaesthesia, and sodium chloride (0.9 per cent) was infused into one or both carotid arteries through cannulae pointing towards the head until the effluent from the jugular vein was clear of blood, then about 1 litre of fixative (40 per cent formaldehyde 5, glacial acetic acid 5, alcohol 72, water 18) was run into the carotid arteries. The hypothalamic and pituitary region was dissected out, embedded in paraffin and cut in serial frontal sections of 10  $\mu$  thickness. Two or three series of sections were retained from each animal, every tenth section from the block being mounted in each series, sets were stained with haematoxylin and eosin, toluidine blue, and by the Bodian [1936] method for nerve fibres

The cells of the supraoptic and paraventricular nuclei were counted under the high power of a binocular microscope using an ocular disc in one eyepiece to give a square field, so that with a mechanical stage it was possible to scan systematically the whole area of the nucleus in any one section. Also the field was subdivided by the disc into smaller squares to facilitate the counting. A high dry objective (magnification  $\times 450$ ) enabled cellular detail to be recognised and only those cells showing a nucleolus were counted. A count was made in each section of the mounted series (every tenth section from the block) so that the sum of the individual counts represented one-tenth of the total number of cells in the whole nucleus. A small error is introduced by the possibility that the knife may split the nucleolus in some cells, a cell so divided would appear in two consecutive sections of the serial sections of which the present count is made in a one-tenth sample. This source of error has been investigated by Jones [1937] and found to be less than 3 per cent.

Characteristically, the cells of the supraoptic and paraventricular nuclei are large and have scanty Nissl material at the periphery leaving the nucleus eccentrically placed in an area clear of Nissl granules. In my sections, whether stained with toluidine blue or haematoxylin and

eosin, many cells showed no Nissl material, although their size, the eccentric position of the cell nucleus, and their situation in the area of the supraoptic or paraventricular nucleus clearly indicated that they were of the same nature as adjacent cells showing Nissl material at the periphery. Cells in the region of the supraoptic and paraventricular nuclei and showing a nucleolus were, therefore, included in the count unless much smaller than the typical cells or unless Nissl material was present in the region of their cell nucleus or throughout the cytoplasm.

There was no difficulty in determining the margins of the supraoptic nucleus even in operated animals with much atrophy, but greater difficulty was encountered with the paraventricular nucleus, where in some animals cells of the same type as those of the supraoptic and paraventricular nucleus extend in close relation to blood vessels from the region of the anterior part of the supraoptic nucleus towards, and sometimes join, the anterior pole of the paraventricular nucleus. These were always excluded from the count, but the anterior margin of the paraventricular nucleus was then ill-defined. Also there is an intermingling of the typical cells with smaller cells of the periventricular system, and in its posterior and lateral aspect the nucleus merges into the posterior hypothalamic area which has large cells but with Nissl material throughout the cytoplasm. Consequently, the margins of the paraventricular nucleus were less easily defined and cells of doubtful morphology were more often encountered.

Several precautions were taken in endeavouring to control the subjective errors. Firstly, duplicate counts were made in some animals using sections stained with toluidine blue and with hæmatoxylin and eosin, the method of staining did not affect the result. Secondly, counts were made in the same animal at intervals as long as two years. Thirdly, since the aim was to estimate the atrophy produced by operative procedures, the sections from operated dogs were examined alternately with sections from normal animals so that any differences in the counting would tend to affect each class equally. In the normal animals, duplicate counts of the cells of the supraoptic nuclei differed by less than 6 per cent and in the operated animals by less than 8 per cent. The greater difficulties in the counting of the cells of the paraventricular nuclei were reflected in less accurate agreement of the duplicates, in normal dogs the difference was always less than 15 per cent and in the operated dogs less than 20 per cent. The figures given in Tables I, II, and III are the means of the duplicate counts.

## RESULTS

*Normal Dogs*—Table I presents the results in six unoperated animals. The total cells of the supraoptic nuclei of the two sides varied between 64,200 and 93,000, the average being 84,900 cells. Rasmussen

[1939] made similar counts and in normal dogs found 35,000-41,000 cells in the supraoptic nuclei on each side, figures which are within the range encountered in the present series

TABLE I—CELLS IN THE SUPRAOPTIC AND PARAVENTRICULAR NUCLEI OF NORMAL DOGS

Weight (kg)	Supraoptic nucleus			Paraventricular nucleus
	Anterior	Posterior	Total	
15 *	24,200	20,000	44,200	8,100
	23,400	24,000	47,400	8,300
			91,600	16,400
11	19,900	24,000	43,900	9,400
	19,700	22,500	42,200	10,200
			86,100	19,600
15	20,000	23,500	43,500	9,600
	20,000	22,600	42,600	9,800
			86,100	19,400
11	16,900	27,700	44,600	5,900
	14,300	29,400	43,700	5,900
			88,300	11,800
16	17,200	14,200	31,400	7,100
	17,600	15,200	32,800	6,400
			64,200	13,500
9	24,400	22,900	47,300	9,500
	22,400	23,300	45,700	10,600
			93,000	20,100

\* Dog, all other animals were bitches

In this and in Table II, in each column the top figure of the pair refers to the left side, the bottom to the right

In the dog the supraoptic nucleus is clearly divided into anterior and posterior portions separated by the optic chiasma and tract the anterior part is a compact mass of cells of the typical morphology lying at the ventral surface of the hypothalamus closely applied to the lateral edge of the optic chiasma and optic tract, while the posterior part is less compact and lies medially and behind the optic tract, between that structure and the fold of the floor of the third ventricle which forms the anterior wall of the pituitary stalk. Very few cells were found forming a connecting band across the upper surface of the optic tract, so that the two parts could be counted separately. There were usually more cells in the diffuse posterior part of the nucleus (average, 22,400 on each side) than in the compact anterior part (average, 20,000), although the proportions in the two parts varied in individual animals.

The paraventricular nuclei of the six normal animals contained between 11,800 and 20,100 cells morphologically similar to the cells of the supraoptic nuclei, and the average was 16,800 on the two sides. I am aware of no other counts of the cells of this nucleus, except that of Pickford and Ritchie [1945], who found 44,000 cells in the paraventricular nuclei of one dog.

*Dogs from which the Posterior Lobe had been Removed*—The supraoptic nuclei of the two sides in three dogs killed four months after post-hypophysectomy contained 18,700, 20,900, and 20,900 cells (Table II),

TABLE II—CELLS IN THE SUPRAOPTIC AND PARAVENTRICULAR NUCLEI OF OPERATED DOGS

	Dog	Weight (kg)	Days after operation	Supraoptic nucleus			Para ventricular nucleus
				Anterior	Posterior	Total	
Removal of the posterior lobe	Tarzan	11	140	4,500	5,300	9,800	2,400
				4,000	4,000	8,000	2,400
						18,700	4,800
	Boxer	10	130	6,000	4,000	10,000	3,400
				6,200	4,700	10,900	2,700
						20,000	6,100
	Cinders	14	140	4,800	5,600	10,400	2,400
				4,800	5,700	10,500	2,200
						20,000	4,600
	Sam	10	42	10,700	8,500	19,200	5,200
				10,300	8,000	19,300	5,100
Section of the hypothalamico hypophyseal tracts						38,500	10,300
	Sally	21	51	6,200	4,100	10,300	3,600
				6,200	4,600	10,800	4,400
						21,100	8,000
	Span	10	110	2,300	1,400	3,700	2,100
				4,100	3,500	7,600	2,200
						11,300	4,300
	Tanner I	9	91	3,600	3,800	7,200	1,000
				1,700	1,700	3,400	400
						10,600	1,400
	Hobo	12	127	2,700	2,700	5,400	1,600
				4,000	3,900	7,900	2,300
						13,300	3,900
	Tanner II	10	121	2,800	1,900	4,700	1,500
				4,800	3,300	8,100	2,200
						12,800	3,700

the average being 20,200, or 24 per cent of the number found in normal animals. In each animal the counts from the two sides did not differ by more than 10 per cent, so that loss of cells occurred equally on each

side Atrophy occurred in both anterior and posterior parts of the nucleus in the three animals after post-hypophysectomy an average of 5100 cells (25 per cent of normal) were found on each side in the anterior part of the nucleus and 5000 (22 per cent of normal) in the posterior part

Each of the above animals was allowed to survive at least four months after operation, but material was also available from two animals killed 42 and 51 days after removal of the posterior lobe ("Sin" and "Sally" in Table II) The supraoptic nuclei of "Sin", killed 42 days after removal of the posterior lobe, contained 38,500 cells, clearly more than were found after post-hypophysectomy but with longer survival periods, in "Sally" 21,100 cells were present 51 days after removal of the posterior lobe It appears then that disappearance of damaged cells may not be complete in animals killed sooner than 50 days after section of the pituitary stalk, and all other observations recorded in this paper have been in animals allowed to survive at least three months after operation

The number of cells of the characteristic morphology in the paraventricular nuclei was also reduced by removal of the posterior lobe (Table II), in the three animals killed four months after post-hypophysectomy 4800, 6100, and 4600 cells were found on the two sides, the average being 5200, or 31 per cent of the mean for the six control animals After removal of the posterior lobe the appearance of sections through the hypothalamus in the region of the paraventricular nuclei was deceptive Many cells were still present, particularly at the posterior end of the nucleus, and in examination under low magnification these appeared to be cells of the paraventricular nucleus In the counting under high power, however, it was seen that the Nissl material of these cells extended throughout the cytoplasm, showing them to be cells of the posterior hypothalamic area and not residual cells of the paraventricular nucleus The counts showed quite clearly that removal of the posterior lobe caused gross loss of the typical cells of the paraventricular as well as of the supraoptic nuclei

*Dogs in which the Supraoptico-Hypophyseal Tracts had been Cut* — In each of four animals submitted to the operation of section of the supraoptico-hypophyseal tracts described by O'Connor [1946], there was greater loss of cells from the supraoptic nuclei than after removal of the posterior lobe, the figures being also given in Table II Whereas after removal of the posterior lobe the average number of cells in the supraoptic nuclei of the two sides was 20,200, in the animals killed three to four months after tract section 10,600–13,300 only were found the average being 12,000, or 14 per cent of the number of cells in normal animals In dogs polyuric after section of the pituitary stalk, Rasmussen [1939] found about 10 per cent of the normal number of cells of the supraoptic nucleus and in rats about 20 per cent In the monkey,

Magoun and Ranson [1939] found 29 per cent of supraoptic cells left after stalk section and 18 per cent after interruption of the supraoptico-hypophyseal tracts more centrally in the median eminence

In normal animals and after removal of the posterior lobe, the number of cells in the supraoptic nuclei of the two sides in the same animal did not differ by more than 10 per cent (Tables I and II), but after tract section greater differences were observed which correlated well with the nature and site of the lesion. Thus, in "Tanner II" the section as described in detail by O'Connor [1946] was incomplete on the right side and more cells survived in the right supraoptic nucleus than in the left (Table II). Always more cells survived on the side where the section was less complete, Magoun and Ranson [1939] report similarly on the results of incomplete sections of the stalk in the monkey.

Separate counting of the cells of the anterior and posterior parts of the supraoptic nucleus revealed, as after post-hypophysectomy, a somewhat higher percentage of surviving cells in the anterior part of the nucleus. In the anterior part of the nucleus in the four animals an average of 3200 or 16 per cent of normal remained, and in the posterior part 2800 or 12 per cent of normal.

There was also a greatly reduced number of cells in the paraventricular nuclei in the four animals after tract section. Again the large cells with uniform distribution of Nissl material remained at the posterior pole of the nucleus, but care was taken to confine the count to cells of the morphology characteristic of the supraoptic and paraventricular nuclei, and only 1400-4300 such cells were found in the paraventricular nuclei after tract section. The average was 3300, 20 per cent of the number found in normal animals.

*Daily Urine Volume and Residual Cells of the Supraoptic Nuclei after Operations in the Pituitary Region*—From time to time before and after operation the animals were kept in metabolism cages and the daily urine volume measured. Each observation was made over a period of at least five days, and during a period of collection the animal was maintained on a standard diet of 8 oz dog biscuits or with some animals 8 oz dog biscuits and 4 oz raw meat daily. Water was allowed *ad libitum*. The post-operative urine volumes charted in Table III are the average of observations made during the period from the end of the fourth week after operation until the animal was killed in the fourth post operative month, and are expressed as a percentage of the daily urine volume (usually about 200 cc) of the individual animal under identical conditions before operation. Thus any temporary post-operative polyuria is excluded, and column 2 of Table III gives the extent of the permanent polyuria which resulted from the lesions described in column 4. In column 3 is given the number of cells which were found in the supraoptic nuclei three months or more after operation. The data are arranged in order of severity of the polyuria, and it is

TABLE III

Dog	Daily urine volume, 5th-20th weeks after operation (per cent of normal)	Residual cells in supra optic nuclei	Operation and post mortem findings
1 Flossie	540	11,700	<i>Attempted removal of the posterior lobe</i> Complete loss of pars nervosa and stalk and extensive destruction of median eminence Only a fragment of pars anterior and no pars intermedia left
2 Nigger	490	9,900	<i>Post hypophysectomy and subsequent tract section</i> Loss of pars nervosa and extensive destruction of median eminence Detached and atrophied stalk ending in relation to small residue of pars intermedia and pars anterior (each about 1/10th of normal size)
3 Hobo	450	13,300	<i>Complete tract section with extensive destruction of median eminence</i> Pars nervosa and stalk atrophied pars intermedia and pars anterior normal
4 Span	230	11,300	<i>Nearly complete tract section</i> Stab wound in median eminence Pituitary findings as for Hobo
5 Tanner II	200	12,800	<i>Nearly complete tract section</i> Stab wound in median eminence Pituitary findings as for Hobo
6 Little Black	190	6,600	<i>Post hypophysectomy and subsequent tract section</i> Loss of pars nervosa and stab wound in median eminence Stalk atrophied but contains a few nerve fibres About half of pars anterior intact but only traces of pars intermedia
7 Towser	180	4,100	<i>Post hypophysectomy and subsequent tract section</i> Loss of pars nervosa and extensive wound in median eminence Stalk atrophied and ends in relation to remains of pars anterior (about 1/6th of normal) Trace only of pars intermedia

TABLE III—continued

Dog	Daily urine volume, 5th–20th weeks after operation (per cent of normal)	Residual cells in supra optic nuclei	Operation and post mortem findings
8 Tarzan	150	18,700	<i>Removal of posterior lobe</i> No pars nervosa stalk intact but shrunken About 1/3rd of pars anterior intact but no pars intermedia
9 Boxer	140	20,900	<i>Removal of posterior lobe</i> Findings as for Tarzan
10 Tanner I	130	10,600	<i>Incomplete tract section</i> Pars nervosa and stalk atrophied but contained many nerve fibres Pars intermedia and pars anterior intact
11 Cinders	130	20,900	<i>Removal of posterior lobe</i> Findings as for Tarzan
12 Spick	90	9,200	<i>Post hypophysectomy and subsequent tract section</i> Small unilateral stab wound in median eminence No pars nervosa Stalk atrophied but contained some nerve fibres Only traces of pars intermedia and pars anterior

apparent that there is no correlation with the number of surviving cells of the supraoptic nuclei. Heinbecker and White [1941] plotted a curve showing increasing polyuria with decreasing number of cells in the supraoptic nuclei, but it is based on estimates rather than counts of the number of nerve cells. In Table III no such relationship can be discerned even if comparison is made between animals with similar lesions: thus fewer cells remained in the supraoptic nuclei of "Tanner I" than in "Hobo" with a much larger polyuria, and a similar discrepancy is observed when the findings in "Spick", "Towser", and "Nigger" are examined.

#### DISCUSSION

It is firmly established that the axons of the cells of the supraoptic nuclei pass along the pituitary stalk to the pars nervosa, the origin of the fibres in the stalk from the supraoptic nuclei has been demon-



strated in silver sections [Pines, 1925, Greving, 1928], and retrograde degeneration in the supraoptic nuclei following lesions of the stalk or pars nervosa is well established. Recent reviewers [Clark, 1938, Ingram, 1939] have accepted that the paraventricular nuclei also contribute fibres to the stalk. Greving [1928] was able to follow axons from the paraventricular nuclei ventralwards to the region of the supraoptic nuclei, but was unable to trace them further into the median eminence and pituitary stalk. In the absence of counts of the cells of the nucleus, retrograde degeneration in the paraventricular nuclei following lesions in the stalk or posterior lobe has not hitherto been well established, so that the acceptance of the passage of fibres from the paraventricular nuclei to the posterior lobe has not been based on strong direct evidence. The counts reported in this paper and those of Pickford and Ritchie [1945] demonstrate conclusively loss of cells from the paraventricular nuclei after section of the pituitary stalk, after tract section in the tuber cinereum there remained 20 per cent and after removal of the posterior lobe 31 per cent of the normal number of cells in the paraventricular nuclei of morphology similar to that of the cells of the supraoptic nuclei (Table II). The detailed post-mortem examination of the hypothalamus of animals from which the posterior lobe had been removed revealed no damage to the hypothalamus either directly or as the result of interference with the blood supply. Similarly, in each dog after tract section the lesion was quite clear of the paraventricular nuclei, and the absence of glial reaction and of obvious loss of cells of different morphology adjacent to the posterior pole of the paraventricular nuclei indicates that the atrophy was not a direct effect of the lesion. There appears no doubt that retrograde degeneration of the cells of the paraventricular nuclei does occur after section of the pituitary stalk, and it follows that axons of cells of the paraventricular as well as of the supraoptic nuclei contribute to the mass of non-medullated fibres passing in the pituitary stalk from the hypothalamus to the pars nervosa of the pituitary. Since in normal dogs there are 16,800 cells of the paraventricular nuclei and 84,900 of the supraoptic nuclei, approximately one-sixth of the fibres of the pituitary stalk may arise from the paraventricular nuclei.

The factors determining survival or disappearance of a nerve cell following section of its axon are not clear and no parallel experiments have been found in the literature. In examining the results of this paper, a correlation is suggested when the number of surviving cells is considered in relation to the distance of the section from the nuclei, as is illustrated by fig 1. Removal of the posterior lobe interrupted the axons of the hypothalamico-hypophyseal tracts at B, about 3 mm distal to the site of the lesion of tract section (between AA in fig 1). At the foot of fig 1 are given the average percentage number of the residual cells of the supraoptic nuclei three to four months after operation in

each case and more cells remained after the more distal section, as was also found by Magoun and Ranson [1939]. In one animal "Scotty", weight 7 kg, where the removal of the posterior lobe was incomplete, a mass of pars nervosa remained in connection with the undamaged pituitary stalk, so that the line of section was approximately that of C in fig 1. In this dog 34,500 cells (41 per cent of normal) remained in

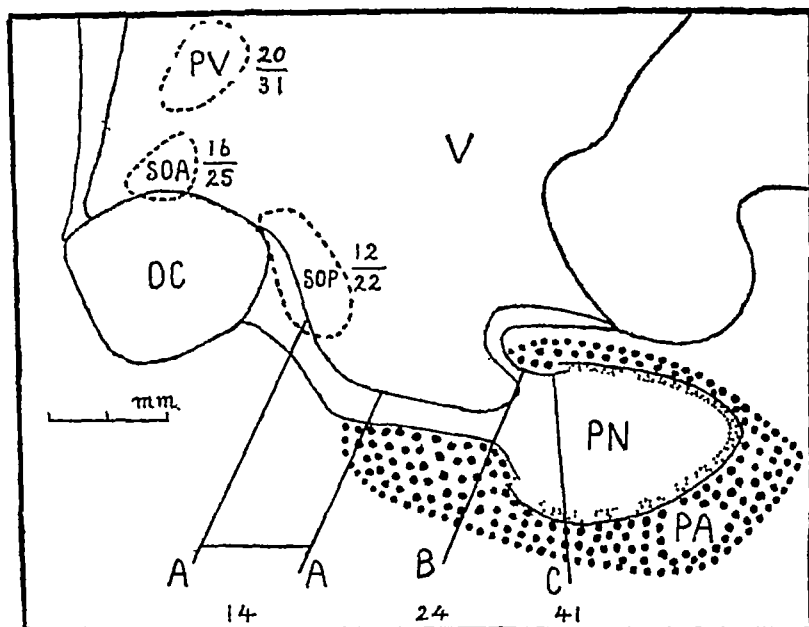


FIG 1—A diagrammatic midline section of the hypothalamic region of a normal dog. OC, optic chiasma; PN, pars nervosa; PA, pars anterior; V, third ventricle; PV is the projection of the paraventricular nuclei on to the midline plane; SOA and SOP, the similar projections of the anterior and posterior parts of the supraoptic nuclei. The lines AA show the site of the lesion produced by the operation of tract section, B the site of the rupture of the stalk in removal of the posterior lobe, C the line of section in "Scotty" where the removal of the posterior lobe was incomplete. The figures at the foot of the frame give the average number of cells found in all parts of the supraoptic nuclei after each operation, expressed as a percentage of the number present in normal dogs. The figures alongside the projections of the nuclei give the percentage of cells which survived in each nucleus. The upper figure of each pair being the average in dogs in which the tracts had been cut at AA, the lower in dogs in which the posterior lobe had been removed with rupture of the stalk at B.

the supraoptic nuclei, still more than after rupture of the stalk at B by removal of the posterior lobe. Further, separate comparison is made in the figure of the extent of the degeneration in the paraventricular and the two parts of the supraoptic nuclei. The distance of the three nuclear masses from the pituitary stalk is illustrated by their projection on to the midline sagittal section, and shows that with section at any level of the stalk, the distance of the section from the nucleus is greatest in the case of the paraventricular nucleus, less with the anterior part

of the supraoptic nucleus and least with the posterior part of that nucleus. In the three animals after removal of the posterior lobe, an average of 31 per cent of the normal number of cells was left in the paraventricular nuclei, 25 per cent in the anterior and 22 per cent in the posterior part of the supraoptic nuclei, after tract section the figures were 20 per cent, 16 per cent, and 12 per cent. By both of these comparisons, then, it holds that the further the lesion from the nucleus the greater the number of cells which survive.

The comparisons in fig. 1 are made using only animals in which the tracts had been sectioned at one operation (3, 4, 5, 10 of Table III). Data are also included in Table III from four animals (2, 6, 7, 12) in which the supraoptico-hypophyseal tracts were cut in the median eminence at a second operation one to six months after operative removal of the posterior lobe. The sites of the final section were similar to those of the single operation on dogs 3, 4, 5, and 10, but only 4100-9900 cells were found in the supraoptic nuclei as opposed to 10,600-13,300 in animals submitted to one operation only. Apparently, if the tracts have previously been severed more peripherally, cells may fail to survive section of their axon at a site at which primary section alone would not have been fatal.

Accepting that the site of section of the hypothalamico-hypophyseal tracts is a factor in determining the degree of atrophy in the paraventricular and supraoptic nuclei, one possible interpretation is that with the more remote sections a greater length of axon remains in continuity with each cell, allowing a greater possibility of survival. For this explanation it has been assumed that all axons of the supraoptic and paraventricular nuclei collect behind the optic chiasma (at AA in fig. 1) and all continue down the stalk to the pars nervosa, so that interruption of the tract at any level results in the transection of the same number of axons, at different distances from their cell bodies. But Vasquez-Lopez [1942] has described nerve endings in the stalk, and in addition many fibres can be seen running in the lateral wall of the stalk between the hypothalamus and the main tract, these are usually regarded as axons arising from the posterior pole of the supraoptic nucleus and joining the hypothalamico-hypophyseal tracts, but may equally include fibres leaving the main tract to re-enter the hypothalamus. It is, then, by no means certain that all fibres from the supraoptic and paraventricular nuclei join and continue in the hypothalamico-hypophyseal tracts across the median eminence and along the anterior wall of the pituitary stalk. A second possible interpretation is that with more distal interruption of the tracts, fewer axons are involved, some having already ended in the stalk or passed out into the hypothalamus, so that the survival of more cells with more distal tract sections may merely reflect the increasing number of axons which have escaped section. Too little is known of the manner of termination of

the hypothalamico-hypophyseal tracts to allow any decision as to the relative importance of these two possibilities

In dogs 1 and 2 of Table III the median eminence, pituitary stalk, and pars nervosa were destroyed, so that there was almost complete loss of the tissues usually regarded as possible sources of antidiuretic substance, suggesting that the 11,700 and 9900 cells which were counted in the supraoptic nuclei of these animals retained very little antidiuretic function. The survival of a cell of the supraoptic nucleus, then, does not necessarily mean that it is able to command the liberation of antidiuretic substance, and it follows that the fact of the survival of 20,000 cells in the supraoptic nuclei after removal of the posterior lobe does not prove that significant liberation of antidiuretic substance may occur from the intact stalk and median eminence. On the other hand, dog 10 provides an instance where tract section was incomplete and some fibres could be followed in silver sections past the lesion into the pars nervosa, so that some of the 10,600 cells which survived in this animal could have retained full normal function. The ability of persistent cells of the supraoptic nuclei to liberate antidiuretic substance may, therefore, range from nil to normal and the count of the residual cells of the nuclei provides no measure of the residual function of the neurohypophysis after operative procedures. Thus, the absence of any relationship between the number of residual cells of the supraoptic nuclei and the degree of permanent polyuria does not in any way compromise the theory that the operative lesion needed to produce diabetes insipidus is the destruction or atrophy of the whole neurohypophysis, including pars nervosa, pituitary stalk, and median eminence—a theory with which in general the post-mortem findings of Table III agree

### SUMMARY

1 Counts have been made of the cells of the supraoptic and paraventricular nuclei of dogs

2 In the supraoptic nuclei of both sides in six normal animals between 64,200 and 93,000 cells were found, the average being 84,900. In three animals killed four months after removal of the posterior lobe of the pituitary 18,700–20,900 cells remained, 24 per cent of normal, in four animals after section of the supraoptico-hypophyseal tracts in the median eminence 10,600–13,300 cells remained, 14 per cent of normal

3 In both paraventricular nuclei of six normal animals there were 11,800–20,100 cells morphologically similar to the cells of the supraoptic nucleus, the average being 16,800. After removal of the posterior lobe 4600–6100 (31 per cent of normal), and after section of the base of the stalk 1400–4300 such cells (20 per cent of normal) remained

of the supraoptic nucleus and least with the posterior part of that nucleus. In the three animals after removal of the posterior lobe, an average of 31 per cent of the normal number of cells was left in the paraventricular nuclei, 25 per cent in the anterior and 22 per cent in the posterior part of the supraoptic nuclei, after tract section the figures were 20 per cent, 16 per cent, and 12 per cent. By both of these comparisons, then, it holds that the further the lesion from the nucleus the greater the number of cells which survive.

The comparisons in fig. 1 are made using only animals in which the tracts had been sectioned at one operation (3, 4, 5, 10 of Table III). Data are also included in Table III from four animals (2, 6, 7, 12) in which the supraoptico-hypophyseal tracts were cut in the median eminence at a second operation one to six months after operative removal of the posterior lobe. The sites of the final section were similar to those of the single operation on dogs 3, 4, 5, and 10, but only 4100-9900 cells were found in the supraoptic nuclei as opposed to 10,600-13,300 in animals submitted to one operation only. Apparently, if the tracts have previously been severed more peripherally, cells may fail to survive section of their axon at a site at which primary section alone would not have been fatal.

Accepting that the site of section of the hypothalamico-hypophyseal tracts is a factor in determining the degree of atrophy in the paraventricular and supraoptic nuclei, one possible interpretation is that with the more remote sections a greater length of axon remains in continuity with each cell, allowing a greater possibility of survival. For this explanation it has been assumed that all axons of the supraoptic and paraventricular nuclei collect behind the optic chiasma (at AA in fig. 1) and all continue down the stalk to the pars nervosa, so that interruption of the tract at any level results in the transection of the same number of axons, at different distances from their cell bodies. But Vasquez-Lopez [1942] has described nerve endings in the stalk, and in addition many fibres can be seen running in the lateral wall of the stalk between the hypothalamus and the main tract, these are usually regarded as axons arising from the posterior pole of the supraoptic nucleus and joining the hypothalamico-hypophyseal tracts, but may equally include fibres leaving the main tract to re-enter the hypothalamus. It is, then, by no means certain that all fibres from the supraoptic and paraventricular nuclei join and continue in the hypothalamico-hypophyseal tracts across the median eminence and along the anterior wall of the pituitary stalk. A second possible interpretation is that with more distal interruption of the tracts, fewer axons are involved, some having already ended in the stalk or passed out into the hypothalamus, so that the survival of more cells with more distal tract sections may merely reflect the increasing number of axons which have escaped section. Too little is known of the manner of termination of

# EXPERIMENTAL OBSERVATIONS ON THE STRUCTURE OF THE BONE MARROW

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RETICULOCYTES are always present in normal blood this fact proves the erythrocytes are not necessarily ripe when liberated into the circulating blood From the standpoint of the interpretation of the reticulocyte formula, it is very important to know whether *all* the erythrocytes achieve their ripening in the circulating blood, or only part of them In other words, are ripe red corpuscles stored in the bone marrow? It is impossible to settle this question by the methods of bone-marrow punctures, because their product contains a mixture of medullary elements and capillary blood Istomanowa [1930], using a technique of rapid cuttings, claimed that *all* the red elements of the marrow were reticulocytes and therefore liberated at this stage, but his method does not avoid the possibility of confusion Koller [1939] claimed to solve the problem on theoretical grounds

## METHOD

The principle of our technique consists in "labelling" visually the red corpuscles of one dog and injecting them into another one under normal conditions Practically we proceed as follows One dog "A" receives intravenously 30 mg phenylhydrazine per kg body-weight According to our experiments [Lambrechts and Nizet, 1943 *a*], the red corpuscles of this dog show, already after a few minutes, a very high ratio of cells containing Heinz's granules (600-900 per cent), when stained post-vitally by brilliant cresyl blue and observed with a dark field condenser [Nizet, 1941, 1943, 1944] The "substantia reticulo-filamentosa" appears yellow, the Heinz's granules brown By our method of observation it is very easy to distinguish both reticulocytes and Heinz's granules, which is impossible by the ordinary microscope The erythrocytes containing Heinz's granules are very well tolerated by the dog during several days and disappear very slowly Therefore this blood may be transfused to another dog and is well tolerated<sup>2</sup> One or two days later, when there is no doubt that the dog "A" has eliminated the excess of phenylhydrazine, we establish a crossed circulation between it and another dog "B" under continuous control

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<sup>2</sup> Such transfusions allowed us to determine with Lambrechts the blood volume of the dog [Lambrechts and Nizet, 1943 *b*]

4 It is therefore concluded that the axons of the cells of the paraventricular as well as of the supraoptic nuclei enter the pituitary stalk and reach the pars nervosa

5 It is suggested that one factor determining the extent of the atrophy of the nuclei after operative section of the hypothalamico-hypophyseal tracts is the site of the lesion, more cells surviving the further the lesion is placed from the nuclei

6 No correlation was observed between the degree of polyuria which resulted from the operative procedures and the degree of atrophy of the supraoptic nuclei, and this is discussed in relation to the residual function of the neurohypophysis and the production of diabetes insipidus

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*Ratio (I), Dog "B"*

Peripheral blood, 0.6776 (on 7000 erythrocytes)

Bone marrow, 0.6762 (on 7000 erythrocytes)

*Experiment II**Donor "A", 4.5 kg* Received i.v. 30 mg phenylhydrazine per kg body-weight the day before*Recipient "B", 3.8 kg* Reticulocytes 2.83 per cent*Chloralose anaesthesia* (1.2 g/kg body-weight)*Blood-pressure during the crossed circulation*

	Dog "A" mm Hg	Dog "B" mm Hg
Before the experiment	200	145
Beginning of the crossed circulation, 10 hr 40 min		
End of the crossed circulation, 11 hr 15 min	200	145
Samples taken at 12 hr		

*Ratio (I), Dog "B"*

Peripheral blood, 0.9402 (on 12,000 erythrocytes)

Bone marrow, 0.9485 (on 12,000 erythrocytes)

In these experiments, the physiological connections of the hæmatopoietic tissues are unchanged and there are only slight circulatory modifications.

It must be emphasised that the number of reticulocytes in the recipient dogs is normal. By our technique we found approximately the same value in normal men [Nizet, 1941, 1944].

## SUMMARY

By means of an original technique it is shown that no ripe erythrocytes are stored in the bone marrow. All the red cells achieve their ripening in the circulating blood.



of the blood-pressure of both. This circulation is maintained for 30 minutes or 1 hour, in order to introduce a sufficient quantity of "labelled" erythrocytes into the blood of "B".

After 24 to 48 hours the plasma of "A" does not contain phenylhydrazine in amounts sufficient to produce Heinz's granules in the blood or bone marrow of "B". This is shown as follows. Phenylhydrazine is active on erythrocytes *in vitro* [Moeschlin, 1941; Nizet, 1945]. Before beginning the crossed circulation, blood samples from "A" and "B" are taken and, after strong centrifugalization, the erythrocytes of "B" are mixed with the plasma of "A". After staining, *no Heinz's granules are found in these erythrocytes. The perfusion of the poisoned blood, therefore, produces no Heinz's granules in the ripe erythrocytes which may eventually be stored in the bone marrow of the dog "B"*.

Ten or 15 minutes after the end of the crossed circulation, a blood sample and several bone-marrow samples (femur, humerus) are taken from the dog "B". Smears and exact counts of the ripe erythrocytes are made, excluding the reticulocytes. The ratio

$$\frac{\text{granular ripe erythrocytes}}{\text{non-granular ripe erythrocytes}} \quad (I)$$

is established

This ratio (I) must be the same in the bone-marrow capillaries as in the peripheral blood if both bloods are well mixed. The bone-marrow sample contains the circulating capillary blood and the fixed medullary elements, red and white. If an appreciable number of ripe erythrocytes were fixed in the bone marrow of "B", the ratio (I) would have a lower value in the marrow samples than in the blood sample (these fixed erythrocytes not being labelled by our process). *We found in our experiments exactly the same value in both*

## RESULTS

We give the results of two experiments

### Experiment I

Donor "A", 5.3 kg. Received *iv* 30 mg phenylhydrazine per kg body-weight the day before

Recipient "B", 3.6 kg. Reticulocytes 4, 2 per cent

Chloralose anaesthesia (1.2 g/kg body-weight)

Blood-pressure during the experiment

		Dog A mm Hg	Dog B mm Hg
Before the crossed circulation		145	155
Beginning of the crossed circulation,	10 hr 39 min		
End of the crossed circulation,	11 hr 15 min	130	150
Samples taken at	11 hr 30 min		

A STUDY OF ARSINE POISONING By G A LEVY (now Imperial  
Chemical Industries Research Fellow in Biochemistry) From  
the Departments of Biochemistry and Pharmacology, University  
of Edinburgh

*(Received for publication 15th August 1946)*

### INTRODUCTION

THIS work on arsine poisoning was carried out in 1940 and 1941 on behalf of the Ministry of Supply. The object was to obtain reliable data for the toxicity of the gas and further information regarding the mechanism by which it causes death.

In studying the toxicity of a gas it is necessary first of all to determine the relationship between concentration and duration of exposure for death in 50 per cent. of a group of animals. For a gas such as arsine, which is irreversibly fixed by the body, it then remains to be found what amounts of the gas are absorbed during median lethal exposures.

Most of the work on arsine poisoning reported in the literature deals with the characteristic blood changes observed after inhalation of the gas. Very little information is available regarding the toxicity of arsine for whole animals, mainly because it has been assumed that the direct and indirect consequences of the reaction with erythrocytes are the sole factors in the lethal action of this gas. It is difficult to see how the relative importance of the various effects contributing to death can be assessed without accurate knowledge of the toxicity of arsine for the whole animal.

The scanty published data for minimum lethal exposures of animals to arsine is scattered through several papers [Dubitzki, 1911, Fuehner, 1922, Joachimoglu, 1920, Joachimoglu and Paneth, 1924, von Oettingen, 1917, Thauer, 1934] and is of little value since either the groups of animals used were very small or the methods of controlling the exposure open to criticism.

In experiments carried out with several species of animals at the Chemical Defence Experimental Station at Porton [Cameron, Carleton, Gaddum, Lovatt Evans and McClurkin, 1939, Fraser, 1940], the concentration of arsine (C) required to cause death in 50 per cent. of a group of animals, after a latent period of some days, was found to vary with the duration of the exposure (T) according to the equation,  $C \cdot T = \text{constant}$ . Low concentrations were thus less effective compared with high than would have been the case if the same amount of arsine

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The arsenic contents of tissues from animals exposed for relatively short periods to arsine have been determined by Joachimoglu [1920] and by Thauer [1934] Thauer concluded that all the arsenic found in organs was derived from hæmolysed erythrocytes The erythrocytes release none of the arsenic fixed during reaction with arsine until they undergo lysis [Thauer, 1934, Cameron *et al*, 1939] The possibility cannot, however, be excluded that part of the arsenic found in the tissues in Thauer's and in Joachimoglu's experiments represented arsine fixed as such before it could react with the red blood corpuscles

In experiments described below, the toxicity of arsine for mice was measured by determining the amounts of arsenic absorbed during median lethal exposures to varying concentrations of the gas As a preliminary step, the median lethal exposure curve for mice already established by the Porton workers was investigated and extended, using an improved procedure for controlling the exposures of the animals to the gas Mice were used as the experimental animals since the whole carcase can be taken for arsenic determination and since they are convenient to work with in statistically significant numbers

The toxicity of arsine for mice was found to reach, under certain conditions, a surprisingly high figure compared with that of arsenites, the most toxic arsenic-containing compounds likely to be formed in the reaction with the erythrocytes This suggested that part of the arsine entering the blood stream may escape reaction with the red corpuscles long enough to reach vital organs and there produce an effect as specific as in the erythrocytes Arsenic determinations were therefore done on tissues, freed from blood before the onset of hæmolysis, from arsine-poisoned rabbits It was assumed that under such conditions any arsenic found in an organ had not undergone previous reaction with the erythrocytes

At this period, the work of Peters, Stocken and Thompson [1945] which showed that dithiols can protect animals against lewisite was in progress It was considered that the possibility should be investigated of protecting animals against arsine by administration of a dithiol A study was therefore made of the effects of ethane-1 2-dithiol, the only suitable dithiol at that time readily available, on arsine-poisoned mice

#### GENERAL CONSIDERATIONS IN GAS CHAMBER DESIGN

In early experiments with arsine, a great deal of information was acquired regarding the effects of various factors in gas chamber design on the results obtained in animal experiments On the basis of this information, a new type of chamber was constructed which permitted accurate control of gas concentration and duration of exposure

In the simple type of chamber commonly used in studying the physiological actions of gases, the gas is introduced after the animals are in place If the exposure is to be prolonged, an air-gas mixture is passed through the chamber With the gas mixture flowing rapidly, the time taken to build up

per kg had had to be inhaled for 50 per cent mortality at all concentrations. There are three possible explanations of this finding.

(1) The effect varies with the amount of arsine absorbed, but the uptake of arsine is not proportional to the product of the concentration and the duration of exposure. This could occur through the animal absorbing a smaller fraction of the inhaled arsine at low concentrations than at high, or through the percentage absorption of inhaled arsine falling off during the exposure. Results obtained by Kiese [1937] in experiments in which he determined the arsine content of inspired and expired air suggest the fraction of the inhaled arsine which is absorbed to remain constant under varying conditions of exposure. Changes in ventilation rate would cause the uptake of arsine to deviate from proportionality to the product  $CT$  while remaining proportional to the amount inhaled. While it is possible that the average ventilation rate may be different in exposures of widely differing durations, it is impossible to conceive changes in the respiration great enough to explain the relationship between concentration and duration found by the Porton workers.

(2) The experimentally derived curve for median lethal exposures of animals to arsine is more truly characterised by an equation of the type  $(C - c)T = \text{constant}$ , where  $c$  is a limiting concentration below which no arsine is absorbed. This explanation may be regarded as improbable in view of the fact that it was found at Porton that animals could be killed by very low concentrations if the exposure was sufficiently prolonged.

(3) The amount of arsine absorbed is a constant fraction of that inhaled, but the former produces effects of varying magnitude according to the conditions of the exposure. If this were true, it would suggest that there exists in the body some mechanism whereby arsine is rendered less toxic sufficiently rapidly to make slight changes in the rate of uptake of great importance in determining the total effect.

By estimating the arsine removed from respired air, Dubitzki [1911] and Kiese [1937] determined the fraction of inhaled arsine which was absorbed. Dubitzki found that cats absorbed 10 to 50 per cent of the arsine inhaled, while Kiese found rabbits to absorb 25 per cent of the gas inhaled. Results obtained in such experiments depend to a great extent on the dead space in the apparatus used. Figures for the arsenic contents of mice killed immediately after exposure to arsine [Fuehner, 1922, Thauer, 1934] suggest a much larger fraction of the arsine inhaled to be absorbed. Kiese found that none of the arsine absorbed by rabbits could be detected in expired air after the exposure.

It has been generally held [Thauer 1934, Lovatt Evans 1941] that practically all arsine absorbed from the lungs undergoes immediate reaction with erythrocytes and that poisoning of the body tissues is caused by the arsenic-containing product or products of this reaction.

slightly and 25 gm zinc shot were introduced After passing through 10N sodium hydroxide solution, the arsine hydrogen mixture was collected in the gasometer

*The Gasometer* —The arsine hydrogen mixture was stored in the gasometer shown in fig 1 Saturated common salt solution was used as sealing fluid, the volume being sufficient to fill the lower bottle completely and cover the two hole stopper in the upper bottle The gasometer found to be most convenient in size was manufactured from 3 litre aspirator bottles, and held 2.5 litres of gas Mounted in a suitable stand, it was easily transportable

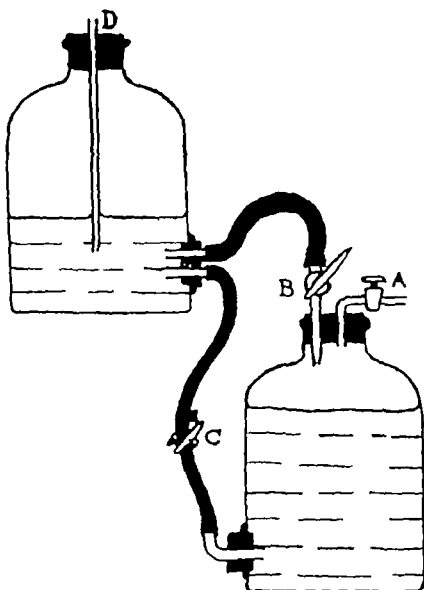


FIG 1 —Gasometer for the collection of arsine and delivery at a measured rate

To prepare the gasometer for the collection of gas, the tube (D) was removed and the screw clip (C) opened When the gasometer was completely charged and the collection of the arsine hydrogen mixture had been discontinued, the screw clip (C) was closed and the stopcock A was opened momentarily to the air to reduce the pressure of the gas to atmospheric

To displace the gas, the salt solution was dropped into the lower bottle through the long handled stopcock (B) The rate of flow of the gas was measured by determining the time necessary for the passage of 10 drops of the solution Calibration of the drop rate in terms of ml/min was done by collecting water displaced from a Mariotte flask in series with the gasometer It was found that, with tube D in position, the volume of gas displaced in unit time varied directly with the drop rate over the range 0.5 to 8 ml/min.

*Sampling the Arsine Hydrogen Mixture* —To determine the arsine content of the gas mixture, a measured sample was shaken in an absorption pipette with N/50 silver nitrate solution Saturated sodium chloride solution was used as sealing fluid in the gas burette After absorption of the arsine was complete, the silver nitrate solution was filtered to remove precipitated silver Solid potassium iodide was added to the filtrate in slight excess of that required to redissolve the precipitate of silver iodide first formed The arsenite in the

the required concentration in the chamber is negligible in long exposures, but cannot be ignored in exposures of short duration. When the exposure is short enough to avoid risk of asphyxiation, the air flow may be omitted and sufficient gas introduced to give the desired concentration when mixed with the air in the chamber. This does not, however, overcome the difficulty encountered in very short exposures in terminating the exposure as abruptly as required. With this type of gas chamber, it is not possible by either procedure to verify the concentration of the gas before exposing the animals.

In an early attempt to construct a chamber permitting short exposures of mice to predetermined concentrations of arsine, a large, inverted vessel resting on a sheet of ground glass was used. The desired concentration of gas was introduced by the open circuit method and checked by analysis before exposing the animals. When the arsine concentration had reached the correct figure, the chamber was slid over the mice, already in place in a small, shallow container sunk below the surface of the sheet of glass. Since the volume of the animal container was a small fraction of the capacity of the chamber, there was very little dilution of the gas when the chamber was moved. The size of the apparatus was limited by practical considerations, and the mice were consequently very crowded. Very erratic results were obtained in mortality experiments with this chamber, due it is believed to the crowding of the animals. The errors may have arisen through the animals rebreathing from an isolated pocket of air and possibly also through removal of arsine from inspired air by filtration through the fur.

Another chamber for the exposure of animals to arsine was constructed on a similar principle. This chamber, details of which are given below, proved to be very satisfactory with widely differing concentrations and durations of exposure. The total volume of the chamber was great enough to allow, without appreciable dilution of the arsine, the use of an animal container holding thirty mice, each in a separate, perforated compartment, or two rabbits. This chamber was used in all the rabbit, and in all but a few of the mice, experiments described in the following pages.

Unless precautions are taken to prevent it, animals tend to huddle together when exposed to arsine. Figures obtained for the arsenic absorbed by mice in exposures in the simple type of gas chamber, in which the animals were free to take up any position they pleased, were lower than those quoted below for similar exposures in the new chamber, in which the mice were kept separate. It seems that when the animals were allowed to huddle together they did not inhale the full concentration of arsine present, although the error was less than that found when the mice were forcibly crowded.

For long exposures, it is considered that, in any type of chamber in which mice are separated from each other, provision should be made for heating. After 5 hours in the new chamber (capacity 140 litres) with pure air flowing in at 2 litres/minute, mice appeared to suffer badly from cold, although there were no subsequent deaths.

## METHODS

*Preparation of the Arsine Hydrogen Mixture*—A saturated, neutral solution of sodium arsenite ( $=20 \text{ mg As/ml}$ ) was prepared from A.R. arsenious oxide. The necessary volume of this stock solution was diluted to 85 ml with water, and 15 ml concentrated sulphuric acid, 3 drops 5 per cent copper sulphate solution and 10 drops 40 per cent stannous chloride in concentrated HCl were added. (If more than 2 mg As/ml were present in the final solution, the addition of stannous chloride was omitted, as otherwise the evolution of hydrogen became uncontrollably violent.) The solution was, if necessary, warmed

by means of a mixing bottle at a rate of from 2 to 6 ml/minute into the air which then passed directly into the gas chamber. The air flow was regulated to dilute the arsine to the desired concentration. Except in very short exposures, the experiments were arranged so that air entered the chamber at a rate of from 1.5 to 2 litres/min (more than sufficient for two rabbits or thirty mice). The air was supplied by a compressor fed from the external atmosphere, and after fluctuations in pressure had been eliminated by passage through a leak valve the rate of flow was regulated by means of a stopcock and measured by a rotameter or flowmeter. The exhaust from the chamber led into a fume duct in which the draught was sufficiently strong to reduce the pressure in the chamber below that of the atmosphere by about 5 mm water. All devices for measuring gas flow were calibrated by water displacement.

When it was found by sampling that the concentration of arsine was steady at the figure desired, the animals in their container were introduced into the chamber and the outer door was locked in place. Further samples of the gas were taken when the animals were in the chamber. When rabbits were being exposed, the dilution of the arsine caused by the introduction of the animals was less than 10 per cent. With mice, the dilution was less than 5 per cent. No great dilution of the gas in the chamber is to be expected, since introducing the animals decreases the total capacity with consequent outward displacement of the gas. Much greater dilution of the arsine occurred when the animals were removed from the chamber since air then entered to fill the space occupied by the container.

The exposures of mice to 0.025 mg  $\text{AsH}_3$ /litre mentioned below were carried out in an ordinary open circuit chamber, constructed from an anatomical specimen jar of 3.5 litres capacity. The jar was enclosed by rods, spaced 7 cm apart, connecting together two brass plates (6.4 mm thick), one of which acted as the door of the chamber. With this chamber, the desired concentration of arsine was built up with the animals in place. The rate of air flow was approximately 1.5 litres/minute, being varied slightly as required for correct dilution of the arsine hydrogen mixture, the latter being introduced at a constant rate of 1.5 ml/min. Under these conditions, the desired concentration was probably reached 5 to 10 minutes after the introduction of the animals. In the prolonged exposures carried out in this chamber, 6 samples of the gas were withdrawn for analysis at regular intervals in each experiment.

*Estimation of the Arsine Concentration in the Chamber*—To determine the arsine content, samples of air were withdrawn from the chamber by means of a suction pump. The air was drawn through a small wash bottle charged with 10N sodium hydroxide solution, an absorption tube packed with glass beads and containing N/50 silver nitrate solution, and a gas meter. The withdrawal of the sample was carried out as slowly as was feasible.

The liquid in the bead tube was allowed to stand for about 15 min, filtered, and after the addition of excess potassium iodide, titrated with iodine, in presence of starch and sodium bicarbonate.

Occasionally, with very high concentrations in the chamber, samples were taken by the procedure used with the gasometer. As a rule, the concentration of arsine in the chamber agreed closely with the figure calculated from the arsine introduced.

#### *Exposure of Mice to Arsine and Determination of the Arsenic Absorbed*

Preliminary experiments suggested that the toxicity of arsine for mice varies for animals drawn from different stocks and belonging to different weight groups. All results given in the following paper were obtained with mice



solution was then estimated by titration with N/200 iodine, in presence of starch and sodium bicarbonate. The absorption pipette and gas burette were similar to those used by Joachimoglu [1920]. Complete determinations carried out in duplicate usually agree to within 3 per cent.

*The Gas Chambers*—The new gas chamber (fig. 2) was constructed from a galvanized iron tank ( $64 \times 51 \times 51$  cm) to which was bolted a sheet of brass, 6.4 mm thick, to form the front face of the chamber. A rubber gasket between the brass sheet and the iron tank ensured an airtight joint. A rectangular aperture,  $53 \times 18$  cm, could be closed by either of two brass doors, 4 mm thick. The doors were hinged separately to either side of the front face of

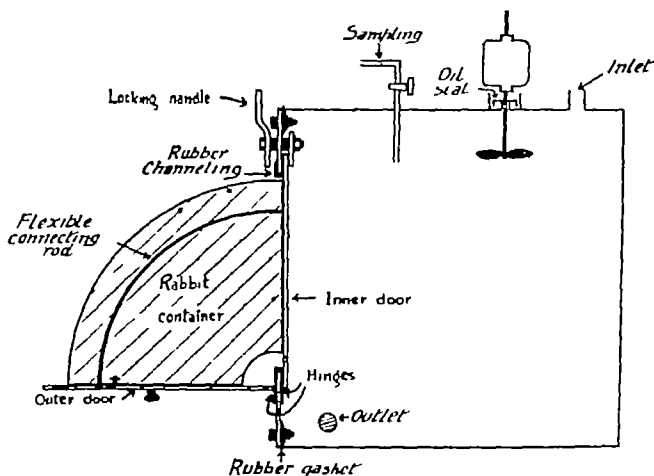


FIG. 2—Diagram showing construction of chamber for accurately timed exposures of animals to predetermined concentrations of arsine

the chamber, and were connected at the unlugged corners, so as to form a right angle, by two, slightly flexible, brass rods (4 mm diameter). The doors thus moved together. When either door was locked in position, it pressed against rubber channelling so as to form an airtight seal. The space between the doors was occupied by the animal container, which was secured to the outer door. A fan, the spindle of which passed through an oil seal, gas inlet and outlet tubes, and a stopcock for sampling completed the chamber.

The empty rabbit container (shown in section in fig. 2) had a capacity of 13 litres against the total chamber capacity of 142 litres. With slight crowding, two rabbits could be got into the container. Exposure of rabbits singly was, however, found preferable. The rabbits were kept in place by horizontal rods fastened to the curved edges of the container. When mice were to be exposed in the chamber, most of the space between the doors was occupied by an airtight dummy resembling the rabbit container. The mouse container, which took the form of a shallow, curved tray of perforated zinc, capacity 4 litres, rested on top of the dummy. The tray was divided into thirty compartments by means of perforated zinc.

Before starting an exposure, the chamber was closed by the inner door. Sufficient arsine was introduced through the sampling stopcock to give approximately the required concentration when mixed with the air in the chamber. Passage of a stream of air containing the correct concentration of arsine was then commenced. Arsine hydrogen mixture from the gasometer was injected

heart was colourless. Heparin (2 mg/100 ml) was added to the first 500 ml saline passed through the animal. Removal of blood from the animal was complete after perfusion for 3 minutes. The blood sample which had been standing at room temperature was at once cooled to 0°C (10 min after the end of the exposure) and examined for hæmolysis. Samples of various tissues were taken and without further treatment analysed for arsenic.

*Treatment of Arsine poisoned Mice with Ethane 1,2 dithiol*—The dithiol was prepared according to the method of Fasbender [1887]. The solubility in water at room temperature was determined iodometrically and found to be 0.4 per cent, permitting the use of aqueous solutions for injection into mice. Solutions in normal saline were prepared immediately before use, as on standing they gave rise to a white precipitate. In practically all cases, injections were intraperitoneal, the volume of fluid injected into each mouse varying from 0.2 to 0.5 ml.

## RESULTS

### *Median Lethal Exposure of Mice to Arsine*

Mortality figures for mice exposed to different concentrations of arsine for varying periods are shown in Table I. Each experiment quoted was carried out with a group of 30 mice, except in the case of

TABLE I—DEATHS IN MICE EXPOSED TO ARSINE \*

Concentration	Duration of exposure	Mortality	Estimated duration for 50 per cent death
mg AsH <sub>3</sub> /litre	mins	per cent	mins
2.5	0.50 0.33	93 20	0.40
1.0	1.25 0.83	57 13	1.18
0.50	10 5 2.5 1.7	100 93 57 0	2.4
0.25	15 9	70 33	12
0.10	70 50	100 50	50
0.025	900 1080 1260 1440 1620 1800	0 0 50 50 50 100	1440

\* 30 mice used in each experiment with 0.1 to 2.5 mg AsH<sub>3</sub>/litre  
 6    "    "    "    "    "    0.025    "    "

drawn from an inbred stock and weighing 25 to 30 gm. Equal numbers of males and females were used in each experiment, but no differences in their response to arsine was ever obtained.

In the study of arsine poisoning carried out at Porton various concentrations of the gas were used in exposures of fixed duration. It was decided, in determining the median lethal exposure curve for our own mice, to work with fixed concentrations of arsine and vary the duration of exposure to each concentration. With this system of working, the experimental error should be less than in the former procedure at those concentrations at which median lethal exposures are characterized by the equation,  $C^2T = \text{constant}$ . Further more, when the concentration is fixed, the results obtained can be readily compared with those for other species of animals by allowing for differences in ventilation rate.

All exposures were carried out with the new gas chamber, except in the case of the prolonged exposures to 0.025 mg  $\text{AsH}_3$ /litre for which the ordinary type 3.5 litre chamber was used. In these long exposures, which were continuous from beginning to end, the mice were provided with food and water.

To determine the arsine absorbed by mice exposed to the gas, the animals were killed by breaking their necks at the end of the exposure. Each carcass was put separately through a household mincer, and 20 gm. of the mixed mince was weighed out on tin, arsenic free paper for arsenic determination. The analyses were carried out by the method previously described [Levvy, 1943].

In determining the arsine absorbed by mice during a 24-hour exposure to the gas, it was necessary to correct for arsenic lost from the body by excretion in the urine. The mice, after weighing, were placed in a small metabolism cage which fitted into the 3.5 litre chamber. At the end of the exposure, they were killed and washed over the metabolism cage so that no urine should be lost, and the combined washings from the cage were analysed.

*Determination of Arsenic in Rabbit Tissues*—In determining the arsenic contents of individual rabbit tissues, it was considered undesirable to wash the blood out of the body by perfusion before killing the animal, since the use of an anaesthetic might affect the course of haemolysis. Two alternative procedures were therefore used to free the tissues from blood before analysis. In both of these, the rabbit was injected intravenously with 10 mg. heparin in 1.0 ml. 0.9 per cent sodium chloride solution immediately after exposure to arsine in the new chamber.

In the majority of experiments, a few seconds after injection with heparin, the rabbit was stunned and the carotid arteries were cut. The blood was collected in a beaker containing a further 10 mg. heparin in 1 ml. saline, cooled to  $0^\circ\text{C}$  and examined for haemolysis. The tissues to be analysed were quickly removed and dropped into ice cold saline. They were then cut into small pieces and washed thoroughly with frequent changes of ice cold saline over a period of one and a half to two hours, to remove as much of the remaining blood as possible. After thorough drying with filter paper, aliquots of the tissues were weighed out for analysis [Levvy, 1943].

In two experiments, the procedure was as follows. After injection with heparin, the rabbit was killed by breaking its neck. The sternum was divided longitudinally and a cannula inserted into the aortic arch above the bifurcation of the left subclavian. A sample of blood was taken from the heart and to it was added 10 mg. heparin in 1 ml. saline. Up to this point the procedure occupied a period of 7 minutes from the removal of the animal from the gas chamber. The rabbit was perfused through the aorta and arterial system with 0.9 per cent sodium chloride solution till the liquid running to waste from the

TABLE II—ARSI NE ABSORBED BY MICE

Concentration (C)	Duration of exposure (T)	C x T	Number of mice in group	Average arsine content (U) and standard error	U/CT (=Vx)
mg AsH <sub>3</sub> /litre	min			mg AsH <sub>3</sub> /kg	
2.5	0.40	1.0	12	0.67 ± 0.04	0.67
1.0	1.18	1.18	11	1.12 ± 0.03	0.95
0.50	2.4	1.2	6	1.28 ± 0.10	1.07
0.25	12	3.0	12	2.44 ± 0.05	0.82
0.10	50	5.0	12	3.50 ± 0.13	0.70
0.025	1440	36	6	10.7 ± 0.75	
				(+ 4.1 mg AsH <sub>3</sub> in urine per kg body weight = 14.8)	0.41

to cause 50 per cent mortality (Table I), it follows that the toxicity of arsine must decrease with decreasing concentration inhaled. The amount of the gas absorbed did not, however, depend solely upon the product of the concentration and the duration of exposure, as can be seen from the last column in Table II.

If C is the concentration of arsine in mg/litre, T the duration of the exposure in min, U the amount of arsine absorbed in mg/kg and V the ventilation rate of the mice during exposure in litres/kg/min, then  $U = CTVx$ , where  $x$  is the fraction of arsine inhaled which is fixed. On rearrangement, the equation becomes  $Vx = U/CT$ . From Table II, it can be seen that  $Vx$  rose with increasing duration of exposure to a maximum at 0.50 mg AsH<sub>3</sub>/litre and then fell considerably. The changes in  $Vx$  account quantitatively for the change in direction of the median lethal exposure curve at 0.50 mg AsH<sub>3</sub>/litre (fig. 3). If the median lethal exposure curve is replotted in such a way as to correct for the variation in  $Vx$ , all the points fall on one straight line, as shown in fig. 4 in which C is plotted against U/C. Since  $U/C = TVx$ , fig. 4 shows the median lethal exposure curve after it has been corrected for variation in  $Vx$ .

The differences in value of  $Vx$  in exposures of varying duration can be explained by assuming changes in V or in  $x$ . It seems more likely that the ventilation rate altered than the fraction of inhaled arsine which was absorbed. If one assumes that  $x$  remained constant, then U/C is proportional to litres of air inhaled per kg body weight during exposure (cf fig. 4). The fall in ventilation rate in exposures of long duration may have been due to the animals falling asleep or suffering from cold or the effects of arsine. In short exposures to high concentrations of gas, the mice may have suspended respiration. The mean value for  $Vx (= U/CT)$  for all the exposures was 0.77. The ventilation

exposures to 0.025 mg  $\text{AsH}_3$ /litre. In these exposures carried out in the 3.5-litre chamber, groups of 6 mice were used. The animals did not appear to suffer unduly from cold in the long exposures in this chamber, and 17 hours' exposure to pure air and hydrogen under the same conditions caused no ill effects.

For periods of exposure ranging from 10 minutes to 24 hours, the results obtained agree closely with the figures found for mice at Porton. Accurately controlled shorter exposures have not hitherto been possible since they required the development of a new type of gas chamber.

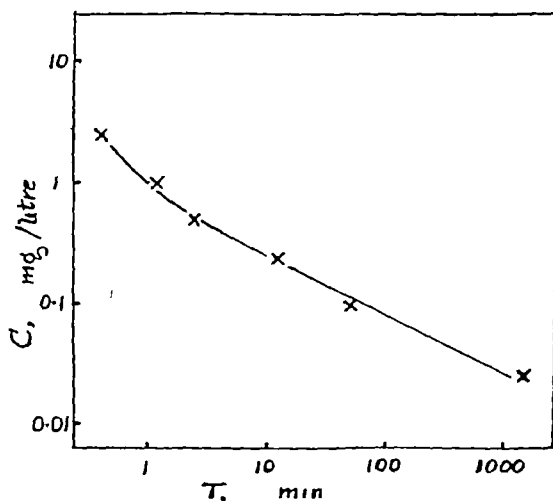


FIG. 3.—Relation between concentration of arsine and duration of exposure for 50 per cent death in mice.

The survival time of the fatalities was more or less the same over the whole range of concentrations studied (2.5 to 0.025 mg/litre) and for varying durations of exposure to any one concentration, the average period before death being 4 days. Hæmoglobinuria was frequently noted at the end of exposures to 0.025 mg  $\text{AsH}_3$ /litre.

At concentrations lower than 0.50 mg  $\text{AsH}_3$ /litre, the relationship between concentration of gas (C) and duration of exposure for 50 per cent death (T) approximates to  $C \cdot T = \text{constant}$  (fig. 3).

#### *Arsine Absorbed by Mice during Median Lethal Exposures*

Figures for arsine absorbed by mice during exposure to varying concentrations of the gas are shown in Table II. In the 24-hour exposure to 0.025 mg  $\text{AsH}_3$ /litre, the figure for the total arsenic in urine was divided by the sum of the weights of the mice used, and the result is shown in Table II in terms of mg  $\text{AsH}_3$  excreted per kg body weight.

The amount of arsine absorbed increased with decreasing concentration inhaled. Since all the exposures were such as had been found

minimum lethal dose of arsenites to be approximately constant at 5 mg As/kg for all species of animals and by all methods of parenteral administration [Fischl and Schlossberger, 1934] Except in the lowest concentrations inhaled, arsine is thus more toxic than arsenites

### *Clearance of Arsine and an Arsenite from Mice*

The clearance of arsenic from mice was studied after exposure to 0.18 mg  $\text{AsH}_3$ /litre for 20 min, or intravenous injection of sodium arsenite in a dose equivalent to 6 mg elemental arsenic per kg body weight. At intervals after treatment, the animals were killed and the arsenic remaining in the body determined as described above. The

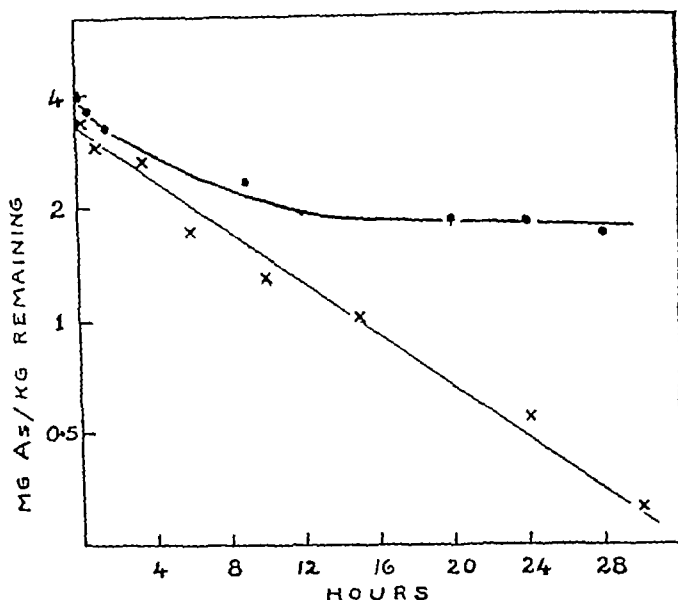


FIG 5—Clearance of arsenic from mice after exposure to arsine or injection of an arsenite

— arsine                      x—x arsenite

results, in terms of mg As/kg remaining, are shown graphically in fig 5, each point representing the average for 3 mice killed at the same time

The clearance of arsenite was exponential since a straight line was obtained by plotting the amount remaining on a logarithmic scale against time on an arithmetic scale (fig 5) and after 24 hours less than one tenth of the arsenic injected remained in the body. In the case of arsine, the clearance followed no such simple course and was much slower, 45 per cent of the arsenic still being present 24 hours after inhalation.

rate for normal mice is about 1.2 litres per kg per min [Gaddum, 1940] Assuming that the mean ventilation rate for all the exposures

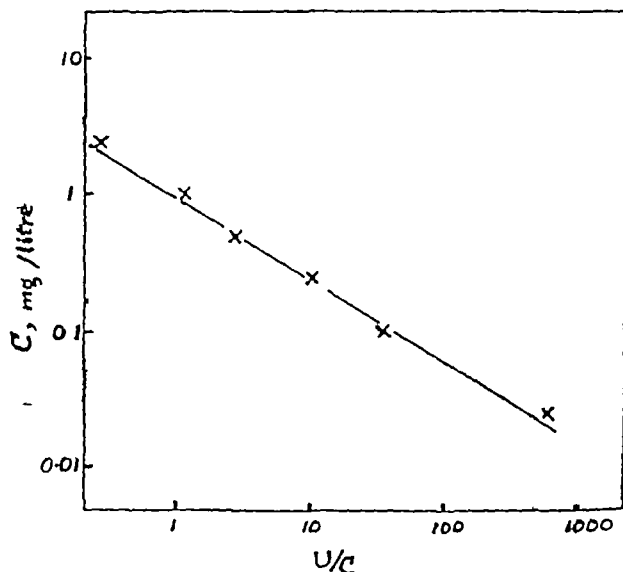


FIG. 4 —The median lethal exposure curve after correction for variation in  $V_x$   
( $U/C = TVx$ )

to arsine approximated to normal, the figure obtained for  $x$ , the fraction of inhaled gas which was retained, is 0.64. This value for  $x$  is of the order one might expect if all expired arsine came from the dead space in the lungs, and hence is quite a probable one.

### *The Median Lethal Dose of an Arsenite*

To assess the toxicity of arsine as determined by the amount absorbed in a median lethal exposure, knowledge of the toxicity of an arsenite was required. Results obtained for the mortality in mice after intraperitoneal injection of sodium arsenite (prepared from A.R. arsenious oxide) suggested the median lethal dose, in terms of elemental arsenic, to be 5 mg/kg (Table III). Figures given in the literature suggest the

TABLE III —TOXICITY OF SODIUM ARSENITE FOR MICE  
ON INTRAPERITONEAL INJECTION

Dose calculated as elemental arsenic

Dose mg As/kg	Deaths
3	0/4
4	1/4
6	3/4
8	4/4
10	4/4

After drying with filter paper, the arsenic content was found to have fallen from 4.6 to 2.0 mg/kg

Since the tissues were weighed out moist for analysis, it was considered advisable to examine the effect of washing for 2 hours with saline on their water contents. The losses in weight undergone by samples of poisoned tissues on drying to constant weight at  $110^{\circ}\text{C}$  were determined, on the one hand with samples taken directly after removal from the animal and on the other hand with samples submitted to the washing process. Of the tissues listed in Table IV, only lung showed a significant difference in the two cases. The ratio of wet to dry weight of lung increased from 4.9 to 8.5 on washing with ice-cold saline.

The results obtained in experiments 4 and 5, in which the animal was freed from blood by perfusion immediately after death, are in general similar to those obtained by the alternative technique after similar exposures to arsine. Slightly higher figures for arsenic in lung were found in experiments 4 and 5 than in 2, 3 and 6, possibly because of the effects of the washing process on the water content of lung tissue in the latter experiments. Since the results obtained in the perfusion experiments (4 and 5) were in general in good agreement with the results in experiments in which the tissues were washed with saline after similar exposures to arsine (2, 3 and 6), it seems that washing the excised tissues did in fact remove the bulk of the arsenic-containing blood.

The concentration of arsenic found in any one tissue does not appear to parallel the blood content, but rather the product of gas concentration and duration of exposure.

As a matter of interest, the total amounts of arsine in the bodies of the rabbits exposed to the gas were calculated according to the method described in a previous paper [Chance, Crawford and Levvy, 1945]. The weight of each tissue in gm/kg body weight was multiplied by its arsine content in mg/gm. Summation of the figures thus obtained gave an estimate of the arsine absorbed in mg/kg body weight. This was then used to calculate the percentage of the gas inhaled on the assumption that the ventilation rate of the rabbits was 0.35 litres/kg/min the value given for normal animals by Gaddum [1940]. These two series of figures are shown at the foot of Table IV.

It can hardly be over-emphasised that the figures thus arrived at have little quantitative value compared with the corresponding figures for mice which were directly determined. It is remarkable, however, that the rabbit figures confirm conclusions arrived at with mice. Over the restricted range studied, the absorption of arsine varied approximately with the product of the concentration and the duration of exposure, and the greater part of the gas inhaled was absorbed (average 60 per cent). According to figures obtained by Fraser [1940] for the



*The Arsenic Contents of Individual Rabbit Tissues*

The animals were exposed for short periods to varying concentrations of arsine in the new chamber. Figures obtained for the arsenic contents of blood, liver, kidney, lung, heart, small intestine, leg muscles and brain are shown in Table IV, the results being expressed as mg arsine per kg moist tissue. In most cases the arsine contents are averages for triplicate determinations. In no case was hæmolysis detectable in the chilled blood sample taken from the animal, nor on incubation at 37° C did the corpuscles commence to hæmolyse till after a period of at least 30 minutes.

TABLE IV—ARSENIC CONTENTS OF RABBIT TISSUES AFTER EXPOSURE TO ARSINE AND PRIOR TO HÆMOLYSIS. RESULTS EXPRESSED IN TERMS OF ARSINE

Number of Experiment	1	2	3	4	5	6	7	8
Weight of rabbit, kg	2.0	2.2	1.8	2.0	2.5	1.9	2.2	2.5
Length of exposure, min	5	20	20	20	20	20	20	20
Concentration of arsine, mg/litre	0.94	0.46	0.46	0.49	0.49	0.50	0.91	0.96
Concentration × time	4.7	9.2	9.2	9.8	9.8	10.0	18.2	19.2
Concentration in blood, mg AsH <sub>3</sub> /kg	6.7	8.9	17.9	16.1	12.9	33.1	42.0	40.0
Concentration in tissues, mg AsH <sub>3</sub> /kg								
Liver	4.6	10.0	9.8	7.5	12.4	7.9	24.2	22.0
Kidney	1.2	4.9	7.7	5.9	4.9	5.1	9.0	8.9
Lung	3.6	4.0	4.4	9.4	6.2	5.3	13.0	9.9
Heart	0.9	2.2	2.5	1.6	1.3	2.3	not done	4.5
Intestine	0.1	1.2	1.0	2.0	2.1	2.0	2.4	2.8
Muscle	0.05	0.4	0.4	0.3	0.3	0.2	0.4	0.7
Brain	none	none	none	0.6	0.5	none	none	0.3
Total AsH <sub>3</sub> found in body, mg/kg	0.68	1.42	2.0	1.96	1.90	3.02	4.40	4.43
U/0.35 × CT, per cent	41	44	62	57	56	86	69	66

In all experiments except numbers 4 and 5, the first procedure described above for removal of blood from the tissues was followed, the rabbit being bled to death and the tissues washed in ice cold saline. To discover if the washing process removed arsenic from the tissues, a sample of poisoned liver, the arsenic content of which had been determined in the usual way, was immersed in saline in the ice-chest for an additional 24 hours, with frequent changing of the wash solution.

required for protection against the effects of a 5 minute exposure to 0.50 mg  $\text{AsH}_3$ /litre, varying doses of the dithiol were injected intraperitoneally 10 to 30 min after the end of the exposure. The results (Table VI) suggest that 17 mg/kg dithiol should reduce the mortality caused by such an exposure to arsine from 100 per cent to 50 per cent.

Injection of the dithiol from 3 hours before to 4 hours after exposure to arsine appears to have been just as effective for protection against the lethal action of the gas as administration immediately after the exposure, as can be seen from Table VII. A considerable reduction in the mortality was still observed when the dithiol was injected 6 to 9 hours after exposing the animals to arsine. The dithiol was, however, ineffective when administered 12 hours after the mice were exposed to the gas.

TABLE VII—EFFECT OF VARYING THE INTERVAL BETWEEN EXPOSURE TO ARSINE (0.50 MG/LITRE) AND INJECTION OF DITHIOL

Time of injection with respect to time of exposure, hours	Total mortality	Mortality, per cent
4 min exposure followed by injection of 15 mg dithiol/kg		
-3	2/10	20
0 to 0.5	3/28	11
+3	1/10	10
+9	3/9	33
+23	6/9	67
5 min exposure followed by injection of 25 mg dithiol/kg		
-2	2/10	20
0 to 0.5	2/10	20
+2 to +4	2/18	11
+6 to +8	10/29	34
+12 to +14	18/19	95

In many of the experiments in which the dithiol reduced the mortality, extensive hæmoglobinuria was noted before the animals were injected. With one group of 8 arsine-poisoned mice injected intravenously with the dithiol, the response was in no way different from that obtained after intraperitoneal injection under similar conditions.

## DISCUSSION

In the introduction, three possible explanations were given of the fact that for 50 per cent mortality animals have to inhale much more arsine at low concentrations than at high. The experiments with mice show the last explanation to have been correct. A practically constant (and large) fraction of the gas inhaled was absorbed at all concentrations, but the toxicity of the arsine absorbed decreased with decreasing concentration inhaled. The body must therefore possess some very effective

mortality in rabbits exposed to arsine under varying conditions, the exposures in all except experiments 4 and 5 were approximately those required for 50 per cent mortality. The calculated figures for mg arsine absorbed per kg in the median lethal exposures are in fair agreement with the corresponding figures in mice for similar concentrations of the gas.

### *The Effect of Ethane-1 2-dithiol on Arsine-Poisoned Mice*

Throughout these experiments the concentration of arsine was fixed at 0.50 mg /litre, the animals being exposed in batches of 20 to 30 in the new chamber. The duration of the exposure was in some cases 4 minutes and in others 5 minutes. Groups of 8 to 10 mice were used for each experiment.

Control exposures of untreated animals to arsine were scattered through the dithiol experiments. The exposures to 0.50 mg  $\text{AsH}_3$ /litre to which the mice were subjected in the dithiol experiments were longer than required for 50 per cent mortality. Figures obtained in studying the toxicity for mice of the dithiol alone are given in Table V. The median lethal dose appears to have been about 55 mg /kg.

TABLE V—THE TOXICITY OF ETHANE 1 2 DITHIOL ON INTRAPERITONEAL INJECTION

Dose, mg /kg	Total mortality	Mortality, per cent
15	0/20	0
40	0/10	0
50	3/9	33
75	10/10	100

That ethane-1 2-dithiol has a beneficial effect in arsine poisoning was found in three experiments in which 15 mg /kg was injected 0-30 min after exposure of mice to 0.50 mg  $\text{AsH}_3$ /litre for 4 min. The total mortality was reduced from 13/20 (65 per cent) to 3/28 (11 per cent). To discover the minimum quantity of ethane-1 2-dithiol

TABLE VI—REDUCTION IN MORTALITY IN ARSINE POISONED MICE PRODUCED BY VARYING DOSES OF ETHANE 1 2 DITHIOL \*

Dose, mg /kg	0	2	4	8	12	15	20	25
Total mortality	19/19	9/9	10/10	5/10	8/10	4/10	5/10	2/10
Mortality, per cent	100	100	100	50	80	40	50	20

\* Dithiol injected intraperitoneally 10 to 30 min after exposure to 0.50 mg  $\text{AsH}_3$ /litre for 5 min

required for protection against the effects of a 5 minute exposure to 0.50 mg  $\text{AsH}_3$ /litre, varying doses of the dithiol were injected intraperitoneally 10 to 30 min after the end of the exposure. The results (Table VI) suggest that 17 mg/kg dithiol should reduce the mortality caused by such an exposure to arsine from 100 per cent to 50 per cent.

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+ 9	3/9	33
+ 23	6/9	67
5 min. exposure followed by injection of 25 mg dithiol/kg		
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0 to 0.5	2/10	20
+ 2 to + 4	2/18	11
+ 6 to + 8	10/29	34
+ 12 to + 14	18/19	95

In many of the experiments in which the dithiol reduced the mortality, extensive hæmoglobinuria was noted before the animals were injected. With one group of 8 arsine-poisoned mice injected intravenously with the dithiol, the response was in no way different from that obtained after intraperitoneal injection under similar conditions.

## DISCUSSION

In the introduction, three possible explanations were given of the fact that for 50 per cent mortality animals have to inhale much more arsine at low concentrations than at high. The experiments with mice show the last explanation to have been correct. A practically constant (and large) fraction of the gas inhaled was absorbed at all concentrations, but the toxicity of the arsine absorbed decreased with decreasing concentration inhaled. The body must therefore possess some very effective

mechanism for detoxicating arsine Unless one were prepared to accept an indefinite rise in the toxicity with increasing concentration of the gas, one would have to assume at some point a change in the character of the median lethal exposure curve The latter was found to occur with mice in very short exposures made possible by means of the new gas chamber, as the concentration was raised beyond 0.50 mg  $\text{AsH}_3$ /litre It appears that the change in direction of the median lethal exposure curve can be explained from the manner in which the ventilation rate varies according to the duration of exposure That the same processes were responsible for death throughout the entire range of concentrations studied is shown by the fact that the average survival period between exposure and death was unchanged

The work done at Porton [Cameron *et al.*, 1939, Fraser, 1940] showed the relationship between concentration of arsine (C) and the duration of exposure (T) required for 50 per cent mortality to be characterised by the equation,  $C^2T = \text{constant}$ , for all species of animals studied It further appeared that the same median lethal exposure curve would serve for all species if the results were expressed on an absolute time scale by correcting for differences in normal ventilation rate If one assumes the same percentage absorption of inhaled arsine for all species, it may be deduced that the median lethal dose in terms of mg absorbed per kg weight should be characterised solely by the concentration of the gas in the atmosphere, irrespective of species differences in the period required for inhalation of the required amount of gas For what they are worth, the calculated figures for arsine absorbed by rabbits support this conclusion More work with other animals than mice is, however, necessary to decide this point

The only factors at present recognised as contributing to the lethal action of arsine are poisoning by arsenic compounds released from haemolysed erythrocytes, blockage of the kidney tubules by haemoglobin and decrease in the oxygen-carrying capacity of the blood [Thauer, 1934, Cameron *et al.*, 1939] All of these effects are secondary to the reaction of arsine with the red blood corpuscles While it may be possible, from these effects only, to explain the high toxicity of arsine in the higher range of concentrations, the possibility should not be overlooked that the gas may produce effects distinct from the direct and indirect ones resulting from its reaction with the red blood corpuscles This has been generally held to be impossible in view of the rapidity of the reaction in blood The fact, however, remains that, for inspired arsine to pass from the gaseous phase to the erythrocytes, it must first enter physical solution in the plasma Compared with that of, for example, oxygen, the solubility of arsine is high, the absorption coefficients in distilled water at 20°C being arsine 0.225 [Jung, 1939] oxygen 0.031 The total circulation time in man is about 25 seconds, and there is no evidence to suggest that the reaction between arsine

and erythrocytes is so rapid, as to make it impossible for part of the arsine dissolved in the plasma to escape fixation by the red blood corpuscles long enough for it to be carried from the lungs to organs such as the liver and kidney

The results of the experiments with rabbits, in which release of arsenic fixed by the erythrocytes was carefully avoided, suggest that unchanged arsine reached liver, kidney and lung cells in far from negligible amounts. In certain organs arsine might be expected to produce an effect as characteristic as that seen in blood, thus in part explaining its potentially high toxicity

It seems probable that at least the greater part of the arsenic found by Kiese [1937, *see also* Heubner, 1937] in the tissues of dogs after chronic poisoning with arsine was derived from hæmolysed red blood corpuscles. On the other hand, it is not unlikely that much of the arsenic found by Thauer [1934] and by Joachimoglu [1920] in the tissues of animals after quite short exposures to arsine was fixed as unchanged arsine

If it is assumed that unchanged arsine has a high toxicity for vital organs and can reach them unchanged, the variation in the toxicity of the gas with the concentration inhaled, *i.e.* with the rate of absorption into the body, may be explained by supposing that as the concentration falls, a smaller proportion of the gas escapes fixation by the red blood corpuscles. The reaction in the blood can then be regarded as resulting in the detoxication of the arsenic-containing molecule from the viewpoint of the body as a whole. The view that an arsenite is the most toxic arsenic-containing compound likely to be found in the reaction between arsine and the erythrocytes receives some support from the contemporary work on this reaction by Crawford, Graham and Marrian [1946]. The contribution by arsenites freed from hæmolysed erythrocytes to the general state of arsenical poisoning, which can never be regarded as insignificant, must become predominant with low concentrations of arsine. Its effectiveness will then be conditioned by the balance between the rate of absorption of the gas on the one hand and oxidation and excretion on the other [*see Chance et al*, 1945].

The duration of exposure to 0.50 mg  $\text{AsH}_3$ /litre required for 50 per cent mortality in mice was 2.4 min. After an exposure of approximately twice this duration, the mortality was brought back to 50 per cent by ethane-1,2-dithiol in a dose of 17 mg/kg. The median lethal dose of the dithiol alone was about 55 mg/kg. Defining the median effective dose as that reducing to 50 per cent the mortality produced by twice the median lethal exposure to arsine, the therapeutic range of the dithiol was thus about 3 (55/17). This figure does not, of course, necessarily hold for the exposure of mice to concentrations of arsine other than 0.50 mg/litre.

The dithiol studied did not appear to react with arsine *in vitro*. It

mechanism for detoxicating arsine Unless one were prepared to accept an indefinite rise in the toxicity with increasing concentration of the gas, one would have to assume at some point a change in the character of the median lethal exposure curve The latter was found to occur with mice in very short exposures made possible by means of the new gas chamber, as the concentration was raised beyond 0.50 mg  $\text{AsH}_3$ /litre It appears that the change in direction of the median lethal exposure curve can be explained from the manner in which the ventilation rate varies according to the duration of exposure That the same processes were responsible for death throughout the entire range of concentrations studied is shown by the fact that the average survival period between exposure and death was unchanged

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is likely, however, that arsine having once entered into combination with cellular constituents, the arsenic may be split off by the dithiol Arsenites released after the reaction between the gas and erythrocytes and possibly also tissue cells will at once combine with the dithiol and thus no longer contribute to the general state of poisoning

### SUMMARY

1 In experiments in which the arsenic in the carcasses of mice exposed to arsine was determined, the percentage of the gas inhaled which was absorbed appeared to be approximately the same (64 per cent) over a hundredfold range in gas concentration

2 The median lethal dose of arsine for mice, in terms of the gas absorbed, increased with decreasing concentration inhaled, suggesting that arsine is rapidly detoxicated in the body. At the highest concentration studied, the median lethal dose was 0.67 mg  $\text{AsH}_3/\text{kg}$

3 The ventilation rate in mice appeared to be different in exposures to arsine of different durations. From this, peculiarities in the median lethal exposure curve may be explained

4 The arsenic in tissues from rabbits killed immediately after short exposures to arsine was determined. Although the tissues were freed from blood before hæmolysis commenced, some organs, in particular the liver, contained considerable amounts of arsenic. It is suggested that this arsenic reached the tissues as arsine unchanged by reaction with the erythrocytes

5 Intraperitoneal injection of 17 mg/kg of ethane-1,2 dithiol doubled the duration of the exposure to 0.50 mg  $\text{AsH}_3$  per litre required for 50 per cent mortality in mice. The median lethal dose of the dithiol alone was about 55 mg/kg. It was effective several hours after exposure to arsine

6 Factors in gas chamber design of importance in carrying out toxicity determinations were studied, and a new type of chamber which permitted accurately controlled exposures of short duration was constructed

7 It is concluded that the high toxicity of arsine in the higher concentration range may in part be due to the action on vital organs of unchanged gas reaching them in physical solution in the blood plasma

The author wishes to express his thanks to the late Professor A. J. Clark and to Professor G. F. Marran for encouragement in carrying out this work, to Professor J. H. Gaddum for suggestions regarding the interpretation of certain of the results, to the late George Abbot and to Charles Bell for technical assistance and to N. E. Condon for the construction of gas chambers. Permission from the Director General of Scientific Research (Defence), Ministry of Supply to publish these results is gratefully acknowledged

**"AVIDITY" OF ANTI-A AGGLUTINATING SERA** By D W H  
BARNES and J F LOUITT (A Report to the Medical Research  
Council from the South London Blood Supply Depot )

(Received for publication 10th November 1946 )

TEST sera for use as blood-grouping reagents (anti-A and anti-B) must fulfil certain conditions. The M R C War Memorandum No 9 suggests that these sera should be of high titre, free of cold agglutinins and of rouleaux-forming property, free of macroscopic fat and, in the case of anti-A sera, able to react strongly with  $A_2$  and  $A_2B$  red cells. The Medical Research Council has established STANDARD SERA against which potential test sera should be checked. A similar "reference standard" has apparently been in use in the U S A [Boyd, 1945].

Other authorities recommend that test sera should also be "avid", that is, capable of agglutinating the appropriate red cells quickly and completely. This is of particular importance in blood grouping by a tile method. In measuring "avidity" the usual standards adopted are those of time—the time for the first appearance of macroscopic agglutinates, or the time for agglutination to be complete, or both when a tile technique is employed. These measurements are not highly accurate, depending as they do so much on subjective impressions.

An attempt was therefore made to measure "avidity" by the completeness of agglutination as judged by performing red-cell counts of the unagglutinated red cells. For this, a tube method was adopted. The results were compared with those obtained using a timing method and a tile technique.

#### METHODS

*Unagglutinated Red cell Count*—Serial double dilutions with normal saline of each serum were made. 1 c c of each dilution was mixed in a test-tube (5 cm  $\times$  1 cm diam) with 1 c c of normal saline and 20 cu mm of whole blood. The tubes were then stoppered and mixed. They were allowed to stand one half-hour or longer and the centrifugation procedure of Dacie and Mollison [1943] carried out. Counts of the unagglutinated red cells after the centrifuged deposit had been finally shaken up were made on a Burkner hæmocytometer chamber. At least 500 unagglutinated cells, or those unagglutinated cells in the whole (7.2 sq mm) of the leukocyte counting area, whichever figure was the less, were counted.





*Time of Agglutination*—A white porcelain glazed tile with well depressions was used. Two drops (0.05 c.c.) of the serum-dilution and two drops of blood diluted with normal saline to give a 10 per cent red-cell suspension were mixed in a depression and the time for the first appearance of visible agglutinates noted.

## MATERIALS

Freshly drawn blood was used in each experiment. One individual of sub-group  $A_1$ , another of sub-group  $A_2$ , provided the bloods for testing. The  $A_1$  individual was selected because her red cells were known to be of *more than average* sensitivity to anti-A iso-agglutinin, the  $A_2$  cells were of *less than average* sensitivity.

The sera tested were all anti-A sera. Two were natural human grouping sera conforming to "standard", two were natural human sera from group O persons specially selected for use as agglutinating sera for "unagglutinable cell counts" by Dacie and Mollison's modification of the Ashby technique, two were natural sera from persons of group O taken at random, and two more were immune sera from the same individuals two weeks after a single intramuscular injection of 5 mg. of group A substance of human origin [Morgan and van Heyningen, 1944], two others were immune human sera of specially high titre from individuals also given a single intramuscular injection of 5 mg. of group A substance, and two were absorbed immune rabbit sera prepared as described by Morgan and Watkins [1945], one a pooled sample of a number of high-titre rabbit sera, and one an individual rabbit serum.

None of these sera was fresh. All had been stored without antiseptic, frozen solid for periods ranging from a few weeks up to two years, mostly a few months.

## RESULTS

### *Anti-A Sera and $A_1$ Red Cells (see Table I and fig. 1)*

*Unagglutinated Cell Counts*—The two "standard" natural human grouping sera 271a and 273a showed low (less than 50,000) unagglutinated cell counts in dilutions 1/1 and 1/1-1/4 respectively.

The two selected natural human sera 021 and  $F_1$  showed similarly low counts over a wider range of dilutions 1/1-1/8 and 1/1-1/16 respectively, and showed some signs of a prozone phenomenon.

The two selected high-titre immune human sera *Tay* and *Bro* before inactivation both showed a double zone phenomenon, in 1/1 dilutions the unagglutinated cell count was moderately low and very low respectively, rising in the subsequent one to three dilutions and falling to another low figure in dilutions 1/32-1/64, in later dilutions still the counts began to rise again. The same sera after inactivation showed

TABLE II—UNAGGLUTINATED RED CELL COUNTS A, RED CELLS (TIMES OF AGGLUTINATION IN BRACKETS)

Serum tested (and titre)	Serum dilutions									
	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	
273a (256)	1,446 000 ( > 180)	2,460,000 —	2,920,000 —							
F <sub>1</sub> (512)	44,800 (95)	80,000 (132)	180,000 (145)	400 000 ( > 180)	890,000 —	1 080 000 —				
Tay (8192)	88,300 (48)	115,000 (54)	114 000 (63)	142,000 (76)	173,000 (88)	374,000 (95)	610 000 (160)	1,220,000 ( > 180)		
Isa <sub>2</sub> (2048)	39,700 (38)	56,000 (47)	93,000 (59)	174,000 (88)	480 000 (147)	740,000 ( > 180)	1 300,000 —	2,300 000 —		
Rabbit Pool a (32768)	— (23)	34 000 (27)	36,000 (33)	28,000 (39)	28,000 (46)	33,000 (60)	51 000 (74)	108,000 (107)	300,000 (158)	
Rabbit No 383 (262144)	—	29 000	27,000	39 000	49,000	83,000	137 000	460,000	926,000	

only a single zone phenomenon, the unagglutinated cell count in the low dilutions 1/1-1/4 and 1/1-1/8 respectively being very high

The two random natural O sera *Isa*<sub>1</sub> and *Sal*<sub>1</sub> gave high unagglutinated red-cell counts even in 1/1 dilutions. The inactivated

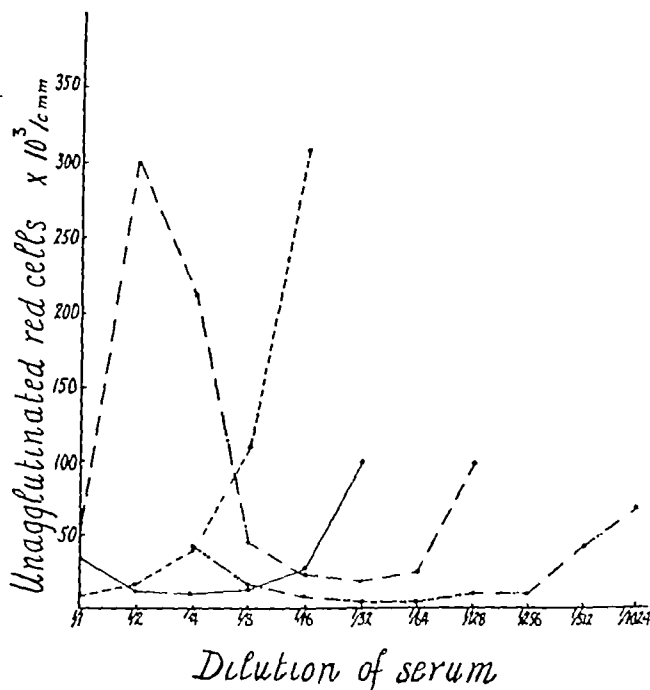


FIG 1—Examples of various types of anti A sera showing the relationship of serum-dilution to strength of agglutination as measured by the unagglutinated red cell count

*A*<sub>1</sub> red cells

- Natural human serum (271a)
- Specially selected natural human serum (*F*<sub>1</sub>)
- High titre immune human serum (*Tay*)
- High titre immune rabbit serum (*Pool a*)

sera *Isa*<sub>2</sub> and *Sal*<sub>2</sub> from the same subjects two weeks later after immunisation gave low unagglutinated red-cell counts over the ranges 1/1-1/32 and 1/1-1/16 respectively. *Isa*<sub>2</sub> was obviously (see Table I) for the purpose a better serum than *Sal*<sub>2</sub> serum.

The pooled immune rabbit serum *pool a* gave low or very low unagglutinated red-cell counts over a very wide range of dilution, 1/4-1/512. Control tests with B and O cells gave no agglutination at any of the dilutions used. The single immune rabbit serum, No 883, gave similar, low or very low unagglutinated red-cell counts from 1/2-1/128, beyond this upper limit it was not tested.

*Agglutination Times*—In contrast with the previous results of the

On the other hand the two rabbit-immune sera gave satisfactorily low counts up to 1/64 and 1/16 respectively

It is notable that zone phenomena were not seen with the  $A_2$  cells except in the case of the pooled rabbit-immune serum, and even here the difference in the figures is of very doubtful significance

*Agglutination Times* —Although these times are longer than in the case of the  $A_1$  cells, the general pattern is the same. The stronger the serum in each case, the shorter the agglutination time

In the case of  $A_2$  cells there did seem to be some suggestive correlation between the degree of agglutination as measured by the unagglutinated cell count and the agglutination time, but it was far from absolute

### DISCUSSION

*The Measurement of "Avidity"* —"Avidity" is usually measured by the time for the appearance of agglutination using a tile technique

The authors consider that the measurement of the time for the appearance of agglutination is inaccurate, as it depends so much on subjective visual impressions. In practice, it is relatively easy to measure when the time is short as the agglutinates then usually increase in size rapidly. Therefore the error is at the most of the order of some seconds. When agglutination is slow, the agglutinates do not increase rapidly and a definite "end-point" is difficult to determine. When a tile technique is used, an agglutination time of 30 seconds or less can certainly be considered as satisfactory

Similarly, the authors consider that the measurement of the time for "complete" agglutination to occur is inaccurate as it depends also on the subjective determination of complete clearing of the serum. This time was not measured in this investigation, but in all instances where such complete agglutination was obtained in the well-tile it was considerably more than the three minutes for which each reaction was observed. It would certainly be easier to measure on a transparent glass slide over a white background as recommended by Coca [1931]

"Avidity", as estimated by the unagglutinated cell counts when the agglutination has been performed in tubes, gives a good measure of the completeness of agglutination under the prevailing conditions. When the counts are low (less than 50,000) the results are readily reproducible. High counts ( $> 100,000$ ), the result of loose or incomplete agglutination, are not quantitatively reproducible, but qualitatively indicate that agglutination is not wholly satisfactory

The marked lack of correlation between the results of the two methods of measurement, at least in the case of  $A_1$  red cells, suggests that different properties of the agglutinating sera are being measured, and that the use of the term "avidity" to cover both the time of agglutination (tile method) and strength of agglutination (tile and tube methods) is



unagglutinated cell counts, the agglutination times were constant in pattern. In the case of each serum the less the dilution of the serum the more rapid was the first appearance of agglutinates. (The time for "complete" agglutination, although not measured, appeared to bear a similar relationship to serum concentration.) Moreover, there was obviously no correlation between the time for agglutination and the degree of agglutination as measured by the unagglutinated cell count.

*Anti-A Sera and A<sub>2</sub> Red Cells (see Table II and fig 2)*

*Unagglutinated Red-cell Counts*—The natural serum 273a even in 1/1 dilution caused only loose agglutination so that there were about  $1\frac{1}{2}$  million unagglutinated cells per c mm.

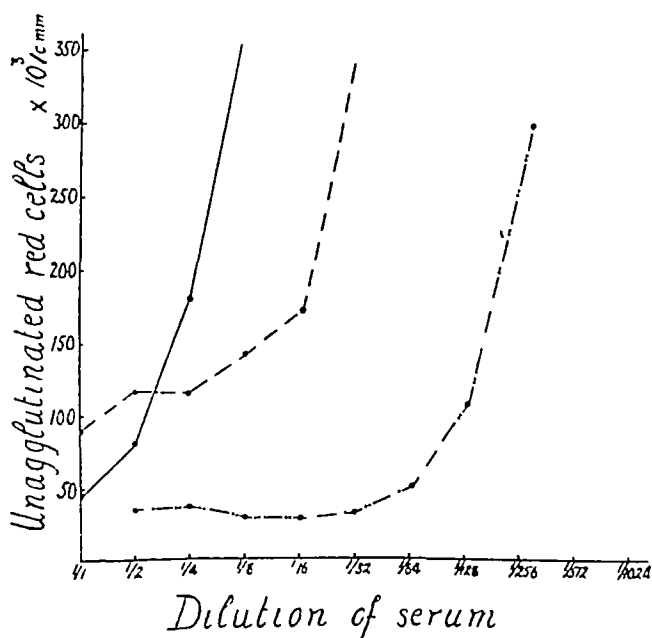


FIG 2—Examples of various types of anti A sera showing the relationship of serum dilution to strength of agglutination as measured by the unagglutinated red cell count

*A<sub>2</sub> red cells*

- Specially selected natural human serum ( $F_1$ )
- - - High titre immune human serum ( $T_{ay}$ )
- · - High titre immune rabbit serum ( $Pool\ a$ )

The natural selected serum  $F_1$  gave markedly better agglutination but only gave low counts ( $< 50,000$  c mm) in the 1/1 dilution.

Of the two human immune sera  $T_{ay}$  and  $I_{sa_2}$  only the latter gave a satisfactorily low count in the 1/1 dilution.

### SUMMARY

"Avidity" of anti-A blood-grouping sera was measured by

- (a) the unagglutinated red cell count,
- (b) the time of agglutination,

with  $A_1$  and  $A_2$  red cells

With each serum agglutination time was always shortest with the lowest dilution. The lowest unagglutinated cell count was with  $A_2$  cell usually in the lowest serum dilution, but with  $A_1$  cells was variable.

Agglutination time and agglutinating strength are therefore probably determined by different factors in serum.

Immune rabbit sera approximated to the ideal anti-A blood-grouping sera rather than natural or immune human sera.

We should like to thank Dr W T J Morgan of the Lister Institute for his help and for liberal supplies of immune rabbit sera.

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unjustified As "avidity" has hitherto been measured by time it is recommended that the term should be restricted to be synonymous with time of agglutination only

The fact that with all the sera investigated the times of agglutination increase progressively with dilution of the sera, suggests that the physical properties of serum as such play a large part in this reaction This may well be related to the phenomenon which permits the rapid slide agglutination test of Diamond and Abelson [1945] and the "conglutination" test of Wiener [1945] in Rh agglutination In both these tests dilution with saline is avoided and the concentration of serum kept equal to whole serum Clumping of the appropriate red cells thereby occurs more rapidly and the clumps, whether they be true agglutinates or not, are massive Pillemar [1945] suggests that cerebroside may play a part in shortening the time of agglutination and the plasma proteins, *e g* albumin [compare Diamond and Denton, 1945] may also be responsible

When the strength of agglutination is measured by the unagglutinated cell counts of  $A_1$  red cells, it is notable that high-titre sera, natural or acquired, frequently show a prozone phenomenon The prozone phenomenon was most marked with the two highest titre-immune human sera *Tay* and *Bro* It is possible that a low-titre univalent non-agglutinating antibody is present in these sera as well as the bivalent agglutinating antibody

*The Selection of the Optimal Hæmagglutinating Serum*—Of the sera tested in this series, those that most nearly approached the ideal hæmagglutinating serum were the two rabbit-immune sera The range of strongly agglutinating power for both  $A_1$  and  $A_2$  cells as measured by the unagglutinated cell count was wide and varied from  $1/2$ – $1/16$  and  $1/2$ – $1/64$  dilutions in the two instances The time of agglutination by the tile method in the case of pool *a* was 30 seconds or less in a dilution of  $1/2$  and 60 seconds or less in dilutions of  $1/32$  downwards These two sera had the highest titre of all the sera tested and in the strengths used showed no non-specific reaction, used diluted they caused no rouleaux-formation, and macroscopic fat and the other constituents of serum causing turbidity were absent In dilutions of  $1/8$ – $1/16$  they would be ideal for tile grouping or tube grouping Rainsford and Morgan [1946] have already reported on the use of this type of serum for the determination of blood groups The immune human sera, although of high titre, tended to have one optimum dilution for tile grouping and another for tube grouping

# THE EFFECT OF BAL ON THE EXCRETION OF ARSEN- OXIDES By AVERIL C CHANCE and G A LEVY From the Departments of Biochemistry and Pharmacology, University of Edinburgh

(Received for publication 7th December 1946)

SHORTLY after their discovery of BAL (British Anti-Lewisite, 2,3-dimercaptopropanol), Stocken and Thompson [1946] showed that application of this substance to the skin hastened the excretion of lewisite in rats. Some time later it was found by Eagle [quoted by Walters and Stock, 1945] that injection of BAL led to a more rapid elimination of arsenic in animals poisoned with arsenicals of therapeutic interest.

In a previous communication [Chance, Crawford and Levy, 1945], the fate of arsenic in the body after injection of rabbits with phenylarsenoxide, mapharsen (*m*-amino-*p*-hydroxyphenylarsenoxide) and the corresponding arsenic acids was described. At the same time as these experiments, others were done in which the effect of BAL on the clearance of the two arsenoxides from the body was studied, but it has not hitherto been possible to publish the results of the latter. A brief account of the work with BAL is given below. It should be noted that it had to be terminated while still incomplete.

## EXPERIMENTAL AND RESULTS

The experimental procedure was described in detail in the previous paper [Chance *et al.*, 1945]. BAL was injected subcutaneously as a 5 per cent (w/v) solution in peanut oil, the dosage being 30 mg/kg in all cases. Doses of the arsenicals, which were injected intravenously, are given in Table I in terms of elemental arsenic. The table shows the amounts of arsenic in the urine and faeces, expressed as  $\mu\text{g As}$  excreted for each mg As injected. When the animals were sacrificed at the end of the seventh day, the lung, liver, kidneys and gall-bladder were analysed for arsenic. Since, at the most, only traces of arsenic were present, the results are not given in detail. Fig 1 shows the cumulative daily excretion of arsenic in the urine in experiments 25 to 28. For comparison, figures from the previous paper for the excretion of arsenic in the urine after the injection of a similar dose of each arsenoxide, but in absence of BAL, are also plotted.



In one experiment (No 20), in which the rabbit received a small dose of mapharsen, BAL had no effect on the rate of excretion of arsenic in the urine, although almost quantitative clearance of arsenic from the body was achieved by the seventh day. However, examination of

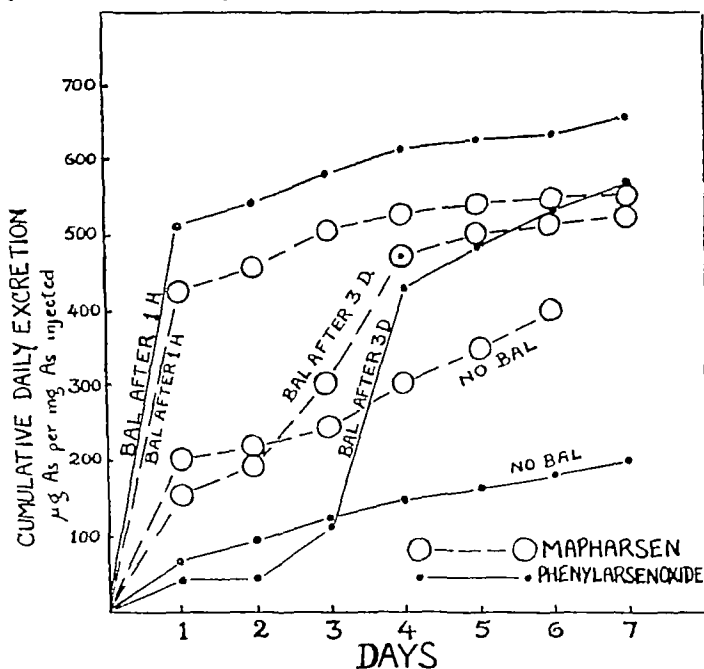


FIG 1—The effect of BAL on the excretion of arsenic in the urine

the results reveals that prior to injection of BAL 90 per cent of the arsenic injected had been cleared from the body, and the fraction in the urine was as high as was ever attained in the experiments shown in Table I. The fact that the remaining 10 per cent was not immediately mobilised by BAL is in agreement with the findings for cases of chronic arsenic poisoning in man [Peters, Stocken and Thompson, 1945]. Although BAL had a pronounced therapeutic effect, it did not alter the slow excretion of the small amounts of arsenic remaining in the body.

### SUMMARY

Administration of BAL (British Anti-Lewisite) hastened the excretion of arsenic in the urine of rabbits injected with phenylarsenoxide or mapharsen, except in one experiment in which only 10 per cent of the arsenic remained in the body at the time of treatment.

Permission from the Director-General of Scientific Research (Defence), Ministry of Supply, to publish this work is gratefully acknowledged.

TABLE I—THE EFFECT OF BAL ON THE EXCRETION OF PHENYLARSENOXIDE AND MAPHARSEN

(Results expressed as  $\mu\text{g}$  As per mg injected.)

Experiment No Arsenical Dose, mg As/kg Period until in- jection of BAL, hours Wt of rabbit, kg	25 Phenylarsenoxide 0.45	26 Phenylarsenoxide 0.46	27 <i>m</i> Amino 3.4	28 <i>p</i> hydroxyphenylarsenoxide 3.4	29 0.42
	1 2.0	72 2.15	1 1.8	72 1.9	72 2.7
<i>Urine</i> Day 1	→ 520	46	→ 430	156	470
2	26	nil	31	54	136
3	40	69	49	97	42
4	30	322	20	168	22
5	15	64	12	32	5
6	7	36	7	19	10
7	23	28	5	12	nil
<i>Faeces</i> Day 1	→ no faeces	nil	→ 128	no faeces	55
2	10	no faeces	74	120	124
3	12	"	22	32	72
4	2	→	16	→	18
5	6	"	2	no faeces	25
6	38	"	14	"	10
7	no faeces	16 7	6	18 93	nil
<i>Total excretion</i>	729	588	815	801	989

## DISCUSSION

Fig 1 shows clearly the effect of BAL on the clearance of arsenic in the urine. It is interesting to note that the final level of urinary arsenic reached was independent of the normal rate of excretion of the arsenic oxide, and of the fraction already excreted when BAL was injected. Twenty-four hours after the injection of BAL, the excretion appeared to settle down to its former rate. Comparison of the results in Table I with those obtained in absence of BAL (previous paper) showed no change in the rate at which arsenic was excreted with the faeces. The total arsenic eliminated in the urine and faeces combined in seven days is shown at the foot of the table. Excretion of all the arsenic injected would be represented by the figure 1000. The effect of BAL on the excretion is reflected in the absence of arsenic from the liver and kidneys in experiments 25 to 28. In corresponding experiments without BAL, these organs contained appreciable amounts of arsenic after seven days.





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# FACTORS INFLUENCING THE PENETRATION OF THE SKIN BY CHEMICAL AGENTS By H CULLUMBINE, M D, Ch B, M Sc (Chemical Defence Experimental Station, Porton, Wilts )

(Received for publication 22nd January 1947 )

EXPERIENCE during the war with the common vesicants, mustard gas and lewisite, suggested that these compounds would be suitable agents for a study of the factors influencing the penetration of chemical compounds through the skin After penetration they produce skin reactions which are easily recognisable and which are capable of a quantitative assessment so that the experimental findings can be subjected to statistical control We have, therefore, used mustard gas—in the liquid and vapour states—and lewisite (in the vapour state) as the vehicles for studying some of the problems of penetration through the skin

## METHODS

### ERRATUM

In a recent paper [Chance and Levvy, this Journal, 1947, 34, 79] the term mapharsen was used to denote the substance metaamino para-hydroxyphenylarsenoxide This term appears to have been first used in this sense by Tatum and Cooper [*J Pharmacol*, 1934, 50, 198] and has been widely adopted The term "Mapharsen" was subsequently registered in the U S A as a trade mark by Messrs Parke, Davis & Co for the hemialcoholate of the above substance In this country the hydrochloride is sold by the same firm under the name "Mapharside" The Editors apologise for any harm that has been done to the interests of the firm by the fact that their name was not mentioned when the term mapharsen was used, and trust that this explanation of the position will set the matter right

time the normal untreated forearms of a large group of subjects to an atmosphere saturated with mustard gas vapour at 30°C These "normal" experiments showed that—

A	1	minute exposure caused an E- reaction	
"	2	" " " " E "	
"	3 5	" " " " E+ "	
"	5	" " " " V- "	
"	6	" " " " V "	
"	8	" " " " V+ "	



TABLE I

Physiological factor	Modification	Method of modification	Skin reaction compared with reaction of normal skin
	NORMAL SKIN		
Circulation	Capillary dilatation	Application of mustard plaster to skin for 1½ hours	No change, though constant tendency to be reduced
		Reactive hyperæmia	
	Capillary constriction	H I 5m liq adrenalin hydrochlor	Increased ( $p=0.01$ )
	Stasis	Sphygmo manometer applied to upper arm and maintained at 200 mm Hg during exposure	No change
Sweating	Increased	H I 0.4 c.c. 2 per cent pilocarpine	Reduced ( $p=0.01$ )
Fat content of skin surface	Reduced	Xylol applied	Increased ( $p=0.05$ )
		Ether applied	No change
	Increased	Adeps Lanæ applied	Reduced ( $p=0.05$ )
Water content of skin	Increased in skin surface	H I 0.4 c.c. normal saline	No change
	Increased on skin surface	Water applied	Increased ( $p=0.01$ )

H I =Hypodermic injection

TABLE II

Local condition of the skin	Mean change in skin temperature	Mean increase in moisture on each sq. cm of skin	Effect on reaction of skin to vesicants
Hot moist	+2.52° C	1.5 mg	Increased markedly ( $p=0.001$ )
Hot dry	+2.75° C	Nil	Reduced ( $p=0.01$ )
Hot normal	+2.26° C	Nil	No significant change though constant tendency to reduce
Normal moist	Nil	2.2 mg	Increased ( $p=0.01$ )
Normal dry	Nil	Nil	No change

That is, the values we have assigned to the reactions occurring in the treated skin should be roughly proportional to the amount of effective mustard gas vapour entering that treated skin

The experiments with lewisite vapour were performed in a similar manner to those for mustard-gas vapour and a similar numerical assessment of the severity of the skin reactions produced was used

In the experiments with liquid mustard gas 0.32 mm diameter drops were placed on the skin—either of the human forearm or of the depilated rabbit abdomen—and the skin reaction noted

The method of statistical analysis used for the results of these experiments was Fisher's method of analysis of variance for Latin squares

In the preliminary experiments some of the physiological properties of the skin were modified in turn and the effect of this modification on the reaction of the skin to the vesicants then determined

In the later experiments the forearms of the subject were placed in a small asbestos lined chamber in which the temperature and relative humidity could be varied between 60° F and 100° F, and between 40 per cent and 100 per cent respectively. The humidities were estimated by means of dry and wet bulb thermometers and a hair hygrometer. Superficial skin temperatures were measured by means of a standardised thermocouple galvanometer. The relative accuracy of the readings was about 0.1° C and the absolute accuracy was about 0.25° C. The moisture on the surface of the skin was assessed roughly by drying with weighed dry cotton-wool swabs. As judged by skin temperature and sweat estimations it was found that it took about half an hour for the skin to come into equilibrium with the atmosphere in the chamber

## RESULTS

Table I summarises the effects obtained when some of the physiological properties of the skin were modified in turn

Similar results were obtained with liquid-mustard gas, mustard-gas vapour and lewisite vapour, and on human and rabbit skin

In general, we can say that the skin reaction is increased by

- (1) capillary constriction,
- (2) defatting with xylol (defatting with ether did not significantly alter the reaction),
- (3) water on the skin

The skin reaction is reduced by

- (1) applying fat to the skin,
- (2) producing sweating by the injection of pilocarpine

In the "atmospheric" experiments the following local skin conditions were produced

- (1) Hot moist skin—a hot, sweating skin, the sweat not evaporating from the skin since the atmosphere was saturated with water vapour
- (2) Hot normal (moisture) skin—a hot sweating skin with normal sweat evaporation occurring

increasing the reaction of the skin to the vesicants, so that neither the lactic acid content nor the acidity of the sweat can be important factors. Again, it can hardly be the fat content of the sweat which enhances its effects, since fat alone reduces the skin reaction.

### DISCUSSION

In a study of the action of mustard-gas vapour, liquid mustard gas and lewisite vapour upon human skin and rabbit skin we have concluded that the reaction of skin to these vesicants is increased by cutaneous capillary constriction during exposure, defatting the surface with xylol before exposure and the presence of water on the skin during exposure, while the skin reaction is reduced by applying anhydrous fat to the skin before exposure and by producing sweating by the injection of pilocarpine. Can we say, therefore, that moisture on the skin or local capillary constriction increases the amount of vesicant penetrating the skin and that fat on the skin decreases the amount penetrating?

The problem is complicated by the fact that, after penetration, some of the vesicant remains in the skin to produce the local reaction while the rest is absorbed into the general circulation. Hence variations in the local circulation, by altering the amount of vesicant remaining in the skin, should alter the local skin reaction. However, the mere application of water to the surface of the skin does increase the skin reactions obtained, and here no alteration in the local circulation is likely to have occurred. Again, a hot moist skin produces a greater skin reaction than does a hot dry skin where the skin temperatures, and presumably the local blood flows, are the same. Hence, moisture on the surface of the skin increases the amount of vesicant that can penetrate the skin and, by a similar reasoning, we conclude that anhydrous fat will produce the reverse effect.

Our experiments give no definite indication as to the influence of the local blood flow on the ability of substances to penetrate the skin. Hyperæmia, produced in a variety of ways (cold, reactive hyperæmia, heat), had no significant effect, though the tendency in most cases was to reduce the skin reaction. In these cases the dilatation of the local skin vessels presents a larger blood-absorbing area so that presumably more of the vesicant penetrating is absorbed into the blood stream and carried away in the skin. [Moritz and Henriques (by communication, 1942, unpublished) have demonstrated, with mustard gas containing radio-active sulphur, that about 90 per cent of the penetrating mustard gas is conveyed away by the circulation, the absorbing of merely another 5 per cent would reduce the amount available for producing local effects in the skin by half.] Conversely, the injection of adrenaline, to produce local capillary constriction and so reduce the vascular absorption, should and did increase the skin reaction. Moreover, as

- (3) Hot dry skin
- (4) Normal (temperature and moisture) skin
- (5) Normal (temperature) dry skin
- (6) Normal (temperature) moist skin

In Table II are given the changes produced by those local skin conditions on the reaction of the skin to the vesicants

It will be seen that a hot moist skin shows the greatest increase in its reaction and a hot dry skin the greatest reduction. The presence or absence of moisture on the surface of the skin would seem, therefore, to be the determining factor. Confirmation of this is obtained from the following experimental facts —

- (1) hot dry skin when moistened with water has its reaction to mustard-gas vapour increased,
- (2) normal skin when moistened has its reaction increased,
- (3) a hot moist skin when dried has its reaction decreased,
- (4) there is no statistical correlation between skin temperature or rise in skin temperature and the reaction to mustard gas or lewisite

Moisture is not the sole factor though, since hot moist skin gives a greater reaction than cool moist skin, and cold moist skin and cold dry skin give the same reaction (see Table III)

TABLE III — EFFECT OF COLD ON THE SKIN REACTIONS TO VESICANTS

Condition of skin	Effect on reaction of skin to vesicants
Cold dry	No change, though constant tendency to be reduced
Cold wet	No change, though constant tendency to be reduced
Normal moist	Increased ( $\rho = 0.01$ )

(The cold skin was produced by applying rubber bags of ice to the forearm for half an hour before exposure to vesicants)

To be sure that we were not under-assessing the effect of heat alone, arms were exposed to a hot dry atmosphere with a temperature of 120° F. The skin reaction was even further reduced, although the skin temperature was raised by 4° to 7° C.

Perhaps sweat on the skin behaves differently from water on the skin? The pH of sweat is about 4.5 and is mainly due to lactic acid. Experiments with aqueous solutions of lactic acid and of hydrochloric acid at pH 4.5 showed that these behave quantitatively like water in

have found that methyl salicylate, camphor and allied substances, when added to anti-gas ointments, increase the decontaminating efficiency of those ointments

### SUMMARY

Using the common vesicants, mustard gas and lewisite, a study has been made of the factors influencing penetration of the skin. It has been concluded that penetration is facilitated by the presence of moisture on the skin and is hindered by the presence of fat. Defatting the surface of the skin will increase penetration, while active sweating with evaporation reduces penetration. The influence of a rise in external temperature is more complex, depending on whether the sweat is allowed to evaporate away and the degree of hyperæmia produced.

### ACKNOWLEDGMENT

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presumably most substances penetrating the skin are absorbed to some extent into the general circulation, we can say that local anæmia will tend to increase and local hyperæmia will probably reduce their effect on the skin

The influence of the activity of the sweat glands would seem to depend chiefly on whether the moisture they produce is allowed to evaporate away or remain on the surface of the skin. With the hot, moist skins the sweat accumulated on the skin, more vesicant penetrated and so a greater skin reaction occurred. The sweat glands of the hot, dry skins were similarly active but yet the skin reactions to the vesicants were much reduced. Moreover, if gross sweating were produced artificially by the injection of pilocarpine then the skin reaction was again reduced.

Why should surface moisture facilitate skin penetration? To answer this question let us consider mustard gas as an example. Ogston in a review (unpublished) of the chemical reactions of mustard gas has pointed out that "there is no evidence that mustard gas reacts *in vivo* by any mechanism other than the substitution of the chlorine atom in an aqueous phase". Hence, it is reasonable to postulate that mustard gas will penetrate and react more efficiently in the presence of moisture than in its absence. The low solubility of mustard gas in water would be no hindrance to penetration, since the ready reaction of the mustard gas in the epithelium would necessitate more of the mustard dissolving in the aqueous phase to keep the latter saturated, and so on. The chemical reactions involving mustard gas in the skin would no doubt be accelerated by temperature in a manner similar to other chemical processes, in addition, the solubility in water will increase with rising temperature. Therefore, we should expect moist skin to give a bigger reaction than dry skin and hot moist skin a bigger reaction still.

As mustard gas is very soluble but relatively inert in fat, a layer of fat on the skin merely interposes a good solvent barrier between the mustard atmosphere and the skin, thus trapping the vesicant in an unreactive phase from which it may later evaporate away. (This argument applies only to anhydrous fat.)

Extreme cold, *e.g.* the application of ice to the skin, by producing hyperæmia and also reducing the rate of reaction of mustard in the skin, we should expect to reduce the skin reaction.

A similar train of reasoning could be applied to any substance whose biochemical reactions chiefly occur in an aqueous phase.

The above experimental findings would form the basis of a logical explanation for the well-established fact that hot and sweaty skin is more sensitive to mustard gas than is cool and dry skin. Again, we should expect rubefacients, by producing a hyperæmia and therefore increased vascular removal of mustard gas, to assist the decontamination of the skin from mustard gas. This indeed has proved to be so, we

THE GLYCOLYTIC ACTIVITY OF THE HEARTS OF VERTEBRATES By JONE J WU and I CHANG From the Department of Pharmacology, the National Medical College of Shanghai, Shanghai

*(Received for publication, 24th January 1947)*

IN a previous communication from this laboratory it was shown that hearts of higher animals such as the rabbit and turtle were more susceptible to asphyxia than those of the lower ones, namely, the frog and the eel. When arranged according to their degree of susceptibility towards asphyxia, they ran in the following order, namely, mammalian heart, turtle's heart, frog's heart and eel's heart [Wu, Yeh, and Chang, 1941]. Thus, while the eel's heart could under conditions of asphyxia maintain an activity for a period not less than five to six hours, the rabbit's heart when deprived of oxygen rapidly declined in activity and became arrested in ten to fifteen minutes.

A number of workers have shown that anaerobically beating heart utilizes carbohydrate and converts it into lactic acid [Nagaya, 1929, Eismayer and Quincke, 1930, Wertheimer, 1930]. Clark, Gaddie, and Stewart [1934] have found that there is a close relation between the anaerobic activity of the frog's heart and the quantity of lactic acid which it produced. Gaddie and Stewart [1934] showed that a frog's heart exhausted of all carbohydrate available for glycolysis could be revived rapidly and completely by glucose or any other substance convertible to lactic acid but not by amino acids and fatty acids. These observations show that the production of lactic acid from carbohydrate forms the most important if not the sole source of energy for the anaerobic heart.

Since the anaerobically beating heart depends upon glycolysis as the most important source of energy, the difference in the anaerobic activity of the hearts of vertebrates would be explicable on the assumption that hearts of lower animals which are resistant to asphyxia have a higher glycolytic activity than those of higher animals which are rapidly depressed by oxygen lack. It is with this assumption in mind that a comparative study of the glycolytic activity of the hearts of vertebrates was carried out in the present series of experiments.

#### METHOD

The hearts of rat, mouse, turtle, toad and eel were used. They were removed from the animal body and placed immediately on ice for five



heart Since it has been shown that the degree of susceptibility towards asphyxia runs in the order of mammalian heart, turtle's heart, frog's and eel's hearts, these results indicate there is some correlation between the glycolytic activity and anaerobic survival of these hearts

2 *The Inhibition of Glycolysis by Iodoacetate*—Iodoacetate is a well-known poison to the glycolytic system It inhibits the activity of the mutases which are essential for the oxido-reductions involved in the Embden-Meyerhof's scheme of muscle glycolysis, namely (1) the conversion of triose phosphate into glycerophosphate and phosphoglyceric acid, and (2) the reduction of pyruvic acid into lactic acid with the simultaneous oxidation of glycerophosphate into triose phosphate or of triose phosphate into phosphoglyceric acid [Green *et al*, 1937]

Although the results of the above series of experiments revealed a definite difference in the glycolytic activity of most of the hearts studied, the difference between the toad's and turtle's heart was almost negligible In order to extend these results, a study of the inhibitory effect of iodoacetate upon the glycolytic activity of the hearts under discussion was made Table II shows that the concentrations of iodoacetate

TABLE II—EFFECT OF IODOACETATE ON THE AVERAGE LOSS OF CARBOHYDRATE ON INCUBATION G PER 100 G OF HEART MUSCLE NUMBER OF ESTIMATES IN BRACKETS

Concentration of iodoacetate	Eel	Toad	Turtle	Rat
1/1,000	0 01 (6)			
1/3,000	0 21 (3)			
1/5,000	0 46 (9)	0 01 (5)		
1/8,000		0 38 (3)	0 02 (5)	
1/10,000		0 53 (2)	0 17 (3)	0 00 (5)
1/15,000			0 30 (2)	0 04 (5)
1/20,000				0 13 (5)
0	1 00 (9)	0 81 (7)	0 76 (10)	0 21 (5)
Initial carbohydrate	3 26 (9)	2 65 (7)	4 86 (10)	1 68 (5)

required for the complete inhibition of glycolysis are for the eel's heart, 1-1000, toad's heart, 1-5000, turtle's heart, 1-8000, and rat's heart, 1-10,000 Since a tissue with a higher glycolytic activity would probably be less readily inhibited by iodoacetate than one with a lower activity, these results in general confirm those obtained in the first series of experiments showing that the glycolytic activity of the hearts under study followed the same order

3 *The Inhibition of Glycolysis by Acid*—Clark, Gaddie, and Stewart [1932] have shown that the anaerobic activity of the frog's heart is dependent upon the amount of buffer present in the system

to fifteen minutes. Each heart was then divided as equally as possible into two to four pieces as might be required by the particular experiment. With the rat's and turtle's heart, these pieces of tissue weighed approximately 100 milligrams each, in this case, one single piece was used for one experiment. With the mouse's, toad's and eel's heart, three or four small pieces taken from a corresponding number of hearts were used in one experiment in order to obtain enough tissue (weighing about 100 milligrams) for accurate chemical analysis. For the control experiments the tissue pieces were analysed immediately for total carbohydrate, while in other experiments they were either placed in Ringer's solution with different pH values or in Ringer's solution with a pH value of 8.4 to which iodoacetate of various concentrations was added. They were then put in an incubator at 37° C, and analysed for total carbohydrate six hours later. The differences in the quantity of total carbohydrate before and after incubation were taken as the amount of glycolysis which had taken place. Ringer's solution was used in the ratio of 1 c.c. of solution to 100 milligrams of tissue. Solutions with pH values of 7.0, 6.5, 6.2, 6.0 and 5.8 were prepared by adding lactic acid to the stock Ringer's solution. Total carbohydrate was determined by Tsai's method [1933].

## RESULTS AND DISCUSSION

1 *The Glycolytic Activity in Alkaline Fluid*—Slightly alkaline fluid provides a favourable medium for the occurrence of glycolysis. Table I shows that when the tissue was incubated at 37° C in Ringer's solution (pH 8.4), the glycolytic activity was highest in the eel's heart, somewhat lower in the toad's and turtle's heart, and lowest in the mouse's and rat's

TABLE I—THE GLYCOLYTIC ACTIVITY OF THE HEARTS OF VERTEBRATE IN ALKALINE FLUID

Species	No of experiments	Total carbohydrate content (g per 100 g)		(A) minus (B)
		(A) Normal	(B) After incubation (6 hrs at pH 8.4)	
Eel	13	3.33 ± 0.06	2.32 ± 0.06	1.01
Toad	11	2.63 ± 0.08	1.84 ± 0.06	0.79
Turtle	14	4.75 ± 0.09	4.01 ± 0.09	0.74
Mouse	8	1.45 ± 0.04	1.25 ± 0.04	0.20
Rat	7	1.68 ± 0.04	1.45 ± 0.04	0.23

hearts of the vertebrates under study in the same order. Hearts which are resistant to asphyxia have a high glycolytic activity, which is not easily inhibited either by iodoacetate or acid. Since glycolysis forms probably the sole source of energy for the anaerobic heart, and a heart with a higher glycolytic activity would be more fit for anaerobic survival than those with a lower activity, these results seem to provide an explanation for the difference in the anaerobic activity of the hearts of vertebrates.

#### SUMMARY

1 The glycolytic activity of the hearts of mammals, turtle, toad, and eel was studied *in vitro*. Under all the circumstances studied, such as the presence of alkali, lactic acid, or iodoacetate, the glycolytic activity is highest in the eel's heart, somewhat lower in the toad's and turtle's heart, and lowest in the mammal's heart.

2 Since glycolysis forms probably the sole source of energy for the anaerobically beating heart, the above results seem to provide an explanation for the difference in the anaerobic activity of the hearts of vertebrates.

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They have found that at a given pH with small volumes of perfusion fluid, asphyxia produces a much more rapid effect than with large volumes of fluid and that with a large amount of alkaline fluid anaerobic activity can be maintained for two or three hours, whereas with an equally large amount of unbuffered fluid, oxygen lack leads to rapid diminution in activity and complete arrest in an hour. Since the anaerobically beating frog's heart depends upon glycolysis as the sole source of energy, it is natural that its activity should be limited by the amount of buffer present, because the lactic acid formed by the anaerobic frog's heart would be excreted into the perfusion fluid and would lower the pH of the latter. When this reaches a certain low value, glycolysis is arrested and the heart deprived of all available sources of energy. The production of lactic acid in the anaerobic heart constitutes therefore an important factor which limits the glycolytic and the mechanical activity of the heart. A series of experiments to study the effect of acid upon the glycolytic activity of the heart tissue was therefore carried out, the chief object of these experiments being to determine the pH value at which complete inhibition of glycolysis took place. Table III shows

TABLE III—EFFECT OF pH ON THE AVERAGE LOSS OF CARBOHYDRATE ON INCUBATION G PER 100 G OF HEART MUSCLE NUMBER OF ESTIMATES IN BRACKETS

pH.	Eel	Toad	Turtle	Rat
5.8	0.00 (8)			
6.0	0.36 (8)	0.00 (4)		
6.2		0.28 (6)	0.00 (6)	
6.5			0.32 (6)	0.00 (5)
7.0				0.12 (5)
8.4	1.02 (8)	0.80 (6)	0.74 (6)	0.23 (5)
Initial carbohydrate	3.68 (8)	2.55 (6)	4.92 (6)	1.67 (5)

that the pH values required for the complete inhibition of glycolysis of the eel's, toad's, turtle's and rat's heart are 5.8, 6.0, 6.2 and 6.5 respectively. The inhibitory effect of acid upon the glycolytic activity of these hearts thus follows exactly the same order as that of iodoacetate, the mammalian heart being most easily inhibited by acid while the eel's heart least. Since the production of lactic acid is a necessary accompaniment of anaerobic activity and probably the only source of energy for anaerobic cardiac contraction, it is natural that the mammalian and turtle's hearts whose glycolytic activity is readily inhibited by acid should be more limited in anaerobic activity than the heart of toad and eel, whose glycolytic activity is less easily affected by acid.

The results of the above three series of experiments all place the

# THE INFLUENCE OF VITAMIN E ON OVARIAN STRUCTURE IN MICE By Z MENSCHIK From the Department of Anatomy, University of Edinburgh, and the Department of Anatomy and Embryology, Polish School of Medicine, Edinburgh

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DETAILED morphological studies of ovarian structure in relation to vitamin E have been made, so far, only on rats, and these have been with reference only to vitamin E deficient diets, and not to diets rich in vitamin E. The results of different investigators vary considerably, most of them failed to find any morphological changes in the ovaries of vitamin E deficient rats. The first authors who made histological studies of the ovaries of rats fed on diets, the composition of which had proved to be vitamin E deficient, were Mattill and Stone (1923), they did not find any changes in the ovaries. Other authors, *e.g.* Evans and Burr [1927], Urner [1931], Juhász-Schaffer [1931], and Stähler, Hebestreit and Fladung [1940], also state that the rat ovary during E-avitaminosis furnished a normal picture. Irregularity of oestrus, and disturbances in ovarian function, in connection with vitamin E deficiency, were observed by Blumberg [1935], Vloek [1938], and Bomskov and Schneider [1939].

Only three papers—Martin and Moore [1939], Bisceglie [1928], Müller [1939]—record morphological changes in the rat ovary during vitamin E deficiency, but the observed changes differed in many points.

The lack of morphological study of ovarian structure in relation to vitamin E in other animals than rats prevents the drawing of any general conclusion regarding the influence of this vitamin on the structure and function of the female gonad. Yet, in general, comparison of the behaviour of different species is of great value in vitamin researches.

## MATERIAL AND EXPERIMENTAL METHODS

The colony of mice was bred in the Department of Anatomy and Embryology, Polish School of Medicine, University of Edinburgh, from a few purchased litters of M R C strain. The experiments were made on 137 females from 39 litters born in the Department. Each litter was divided as evenly as possible between the diets used during the experiments. If a litter consisted of two females only, the vitamin





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E deficient and control diets had priority. The number of animals on each diet examined during three age-periods is shown in the Analysis of Results.

Five diets were used during the experiments.

1 Diet "N"<sup>1</sup> was a stock, laboratory standard diet modified slightly from Thomson's diet [1936], fresh lettuce being the main natural source of vitamin E in a moderate dose. Breeding animals, stock and control animals were kept on diet "N".

2 Diet "B"<sup>2</sup> was a vitamin E deficient diet [see Emerson and Evans, 1937, McCollum, Rask, and Becker, 1928]. In preparing this diet, weighed starch was cooked and mixed, after cooling, with the remaining constituents. The simultaneous presence of cod-liver oil and lard would inactivate possible traces of vitamin E in this diet [Cummings and Mattill, 1931, Mattill, 1940, MacKenzie and McCollum, 1941].

Diet "B" was tested biologically for absence of vitamin E. In a preliminary experiment, all five mice receiving the diet showed typical resorption-gestations.

3 Diet "K"<sup>3</sup> was identical with diet "B", the sole difference being that mice fed on diet "K" received synthetic alpha-tocopherol.

<sup>1</sup> Diet "N"—Stock and control diet

14 per cent dried milk rat cake, manufactured by The North Eastern Agricultural Co op Soc Ltd, Aberdeen

Wheat offal (fine bran)	17.7 per cent
Ground wheat	17.7 " "
Sussex ground oats	17.7 " "
Dried skimmed milk	14.0 " "
Ground barley	8.8 " "
Ground maize	8.8 " "
Meat and bone meal (50 per cent protein)	8.8 " "
White fish meal (64 per cent protein)	4.5 " "
Dried yeast (40 per cent protein)	1.2 " "
Salt	0.4 " "
Cod liver oil	0.4 " "

Fresh lettuce (twice weekly)

Fresh milk (twice weekly) or tap water (remaining days of week)

<sup>2</sup> Diet "B"—Vitamin E deficient diet

Vitamin E deficient fat rich cake, prepared every 10 days in the Laboratory

Casein (Argentine, 90 mesh)	27.0 per cent
Wheat starch	35.0 " "
Pure lard	22.0 " "
Dried brewer's yeast	10.0 " "
Salts, McCollum No 185	4.0 " "
Cod liver oil	2.0 " "

Tap water

<sup>3</sup> Diet "K"—Vitamin E rich diet

Vitamin E deficient fat rich cake, the same as in diet "B" supplemented (except on Sundays) with

Synthetic Vitamin E, alpha tocopheryl acetate solution in olive oil ('Ephynal' Roche) in daily dose of 2.5 mg of alpha tocopherol (0.05 c.c.), solution was administered orally by means of a micro syringe

Tap water



diet "K" prolongs the ovulatory function of the ovary in mice and maintains it when this function normally begins to decline with increasing age

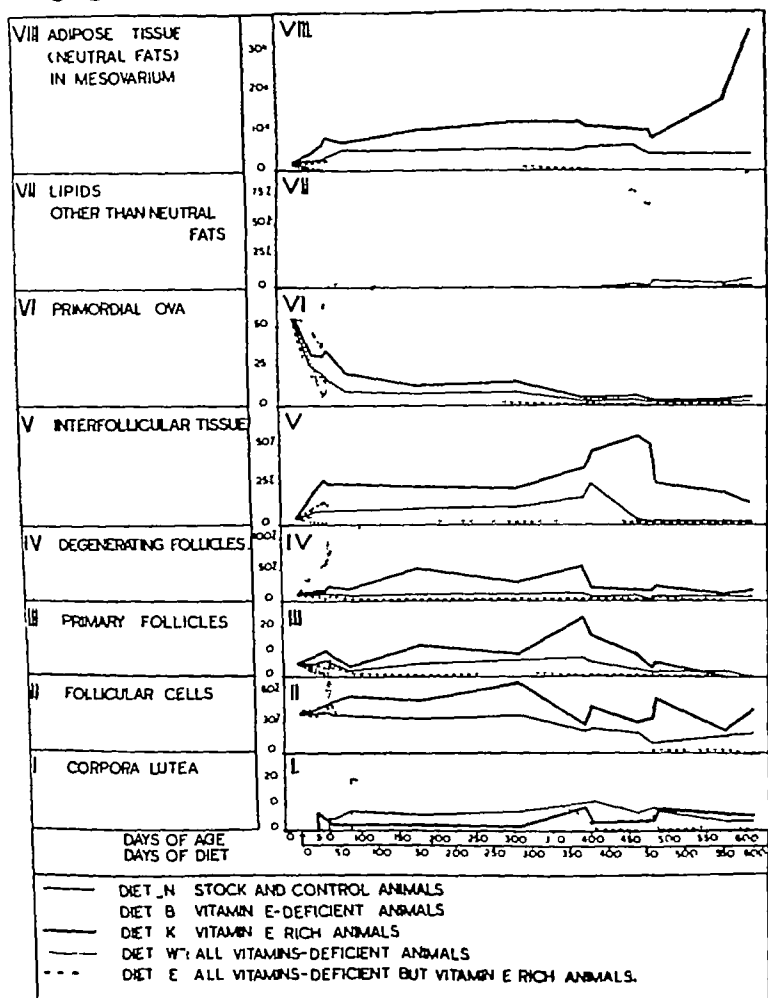


Fig 1

Females on diet "W" (all vitamins deficient) and diet "E" (vitamin E rich, all other vitamins deficient) die before reaching sexual maturity

*Follicles of all Kinds*—The total number of follicles of all kinds does not show any substantial difference on diets "N," "B", and "K". Yet, on diet "B" the follicles are poor in follicular cells, and on diet "K", on the contrary, each follicle is rich in these cells

*Follicular Cells* (fig 1, II)—On all diets the amount of follicular cells keeps on an almost uniform level till approximately the 300th

Serial sections were made of the ovaries of animals, fed on appropriate diets, which were killed or had died after 7, 21, 29, 35, 63, 141, 179, 196, 270, 272, 369, 371, 438, 459, 462, 533, 553, 587, 611, 633, and 791 days of diet. For the purpose of comparing the experimental results, animals of the same age, mainly litter-mate sisters, and after exactly the same periods of different diets, were taken for examination. They were killed by a large dose of ether administered by inhalation.

After a macroscopic examination, the ovary of one side was fixed in Bouin's fluid and embedded in paraffin-wax, and complete serial sections 10 microns thick were made. The results are based on 68 complete series. The ovaries of the other side were fixed in 4 per cent buffered formaldehyde-saline solution [Carleton and Leach, 1938], and sections were made by the freezing microtome for the study of lipids in the ovarian tissue.

Sections were stained by different methods in order to show not only the general structure of the organ, but also the behaviour of connective tissue, lipids, and other structural details. Sections were mounted in euparal, canada balsam, or glycerol jelly.

### EXPERIMENTAL RESULTS

The results of the experiments are diagrammatically represented in fig. 1, and show progressive course of characteristic changes, each main structural part of ovaries is represented separately and estimated quantitatively.

*Sexual Maturity and Corpora Lutea* (fig. 1, I) —Female mice on diet "N" (control) show corpora lutea, an index of sexual maturity, on the 60th day of life on an average, which corresponds to the findings of Mirskaia and Crew [1930 a, 1930 b]. The number of corpora lutea remains throughout life roughly on the same level, but declines after the 500th day.

Females on diet "B" (vitamin E deficient) attain sexual maturity usually a little later—approximately 30 days later—than controls, and they show more corpora lutea, the increase in corpora lutea is due rather to persistence—corpora of different ages can be found in the sections—than to the rupture of more follicles at one estrus. After 300 days of diet "B" the number of corpora lutea falls rather abruptly, so that the presence of corpora lutea after 400 days on this diet is very rare.

Females on diet "K" (vitamin E rich) attain sexual maturity slightly earlier—15 days earlier on an average—than control animals. Although each corpus luteum is usually a little larger in comparison with controls, their number is, for a long time, less than in control animals, only after 450 days on diet "K" does it surpass slightly the number of corpora lutea in controls. It seems, therefore, that

*Interfollicular Tissues* (fig 1, V) —This tissue in the mouse ovary is of germinal epithelium origin (Brambell, 1928] On diet "N" the interfollicular tissue is in moderate amount, slowly increasing with age to the 400th day, and then suddenly falling to a very small amount

Diet "B" interferes with the development of interfollicular tissue to such an extent that the curve runs not far from zero

Diet "K" markedly stimulates the germinal epithelium to produce interfollicular tissue The maximum of the curve falls between the 350th and 450th days of vitamin E rich diet At this age all groups of mice demonstrate an increase in the amount of interfollicular tissue, very pronounced on diet "K", though only moderate on other diets

On diet "W" the interfollicular tissue rapidly disappears

On diet "E" the amount of this tissue, so long as the animals live, is greater than on control diet "N"

As the interfollicular tissue is more abundant on diets "K" and "E" (vitamin E rich diets) than in controls, and on the contrary disappearing on diets "B" and "W" (vitamin E deficient diets), the conclusion seems to be justified that vitamin E stimulates the formation of interfollicular tissue

*Primordial Ova* (fig 1, VI) —The number of primordial ova observed in ovaries declines normally with age The diagram demonstrates, however, that on vitamin E rich diets ("K" and "E") the number is always higher, and on vitamin E deficient diets ("B" and "W") always lower, than on the control diet ("N")

*Lipids other than Neutral Fat* (fig 1, VII) —Besides a minute amount of lipids which make a periodical appearance in corpora lutea [Deansley, 1930, Fekete, 1941], some lipids, other than neutral fat, appear in normal, diet "N", ovarian tissue in a very small amount, but in ageing mice only and not earlier than 400 days

These lipids, however, appear in very large quantity on diet "B" They are formed as early as the 50th day of the diet approximately, and their amount progressively rises so constantly that, after the next 500 days of the diet "B", lipids other than neutral fat account for almost 95 per cent of ovarian content The rise of the curve is most steep between the 300th and 450th days of the diet

In animals on diet "K" a minute amount of these lipids was found only in two animals above 570 days of age, when the ovaries of control animals showed a small, but obvious amount of these substances

Lipids other than neutral fat were not observed in animals on diets "W" and "E"

These lipids, so characteristic for diet "B" (vitamin E deficient), can be demonstrated not only in sections made by the freezing microtome method, but also by the paraffin-wax technique, they are, therefore, not soluble in alcohol, benzene, and xylene (xylol) Attempts to dissolve these lipids in ether, chloroform, acetone, absolute alcohol

day of life (280th day of diet), and it then gradually declines, due chiefly to a decrease in the number of follicles in general, in animals approaching old age

In animals on diet "B" the total amount of follicular cells is about half the amount in control animals and declines with age almost parallel with the corresponding line of diet "N"

Diet "K" induces an increase of total amount of follicular cells in comparison with the control diet. The peak of the curve occurs approximately on the 300th day of age (280th day of diet), thereafter the curve subsides irregularly, less steeply in general, than on diets "N" and "B"

Animals on diets "W" and "E" show an increased number of follicles and consequently of follicular cells also, but the majority of follicles undergo necrotic changes. The amount of follicular cells on diet "E" is slightly higher than on diet "W"

*Primary Follicles* (fig 1, III) —On diet "N" the number of primary follicles, after a long run on an almost even level, gradually declines as the animal approaches old age

Animals on diet "B" show rather quickly—after a few days only on this vitamin E deficient diet—fewer primary follicles than controls. After 65 days of diet "B" the number of primary follicles is very small, and it remains so until about the 500th day of the diet when it declines to zero

Animals on diet "K" show an increased number of primary follicles. The curve goes up gradually, till the 400th day of age (380th of diet), then falls rather steeply to reach zero about 100 days later

Animals on diets "W" and "E" lose promptly the ability to produce new primary follicles

*Degenerating Follicles* (fig 1, IV) —On diet "N" the proportion of follicles undergoing atretic involution (Engle, 1927] is low, and keeps more or less the same level during the whole life

On diet "B" this proportion is of smaller value than in controls

On diet "K" the proportion of follicles which undergo involution—the type of change is closely similar to the normal phenomenon on diet "N"—is very high, reaching almost 50 per cent between the 150th and 350th days of the diet. During prolonged diet "K" (beginning from 465th day of the diet) hæmorrhages into follicular cavities are often observed, a phenomenon which does not occur on any of the other diets

In the diagram, the curves for diets "W" and "E" are rising, but in this case one deals with normal atretic involution in a small degree only and mostly in the first days of the diet, necrosis of follicles is the main feature. The lack of marked difference between diets "W" and "E" seems to prove that vitamin E, acting independently, has little importance, if any, for keeping the follicles healthy



rich diets, on prolonged administration of these diets they show (c) very marked differences in comparison with controls

(a) *Animals 4 Weeks old to 11 Weeks old kept 1 Week to 8 Weeks on Appropriate Diet*

*Diet "N"*—The ovaries of these eight control animals are typical of the ovary of the normal animal. From 8 weeks of age the animals are sexually mature and some corpora lutea are present, follicles are in various stages of development and a few of them are atretic (Pl I, fig 2). Primordial ova are numerous owing to the young age of the animals. Cords of interfollicular tissue, composed of a few layers of epithelioid cells, can be found almost constantly between the follicles. The medullary part of the ovary is small. In the mesovarium, a moderate amount of adipose tissue is seen.

*Diets "B" and "K"*—The ovaries of six vitamin E deficient animals (diet "B") have no corpora lutea in contrast to animals on diets "N" and "K" which possess them, the latter fewer, but the other constituents exhibit almost any deviation from the normal structure of the ovaries of control animals.

The ovaries of eight vitamin E rich animals (diet "K") show few corpora lutea, which made their appearance on an average a fortnight earlier than in controls. There are slightly more follicles, and they are richer in follicular cells than those of animals on diets "N" and "B". There are also more primordial ova. Interfollicular tissue is much more abundant than in diets "N" and "B", and it is present not only in the form of cords between follicles but also as clusters of this tissue (Pl I, fig 3). In the mesovarium, there is approximately twice the amount of adipose tissue formed in normal animals of the same age.

*Diets "W" and "E"*—Ovaries of the nine animals fed on diet "W" are very small, and consist of connective tissue and of many, usually small, follicles most of which show a colliquative necrosis (Pl I, fig 4). There are very few primary follicles and primordial ova, and almost all the ova have abnormal granular cytoplasm with ill-defined outline of the nucleus. Interfollicular tissue is absent. In the mesovarium no fat can be found.

Ovaries of the eleven animals fed on diet "E" show changes resembling animals kept on diet "W", but with the following main differences: follicles contain more follicular cells, interfollicular tissue is present though in moderate amount, there are more primary follicles than in diet "W" and some of them are normal in appearance, and, what is most conspicuous, there are very many primordial ova (Pl I, fig 5) most of them showing all details of normal structure.

and ether in equal parts—14 hours in each solvent—showed that their solubility is very limited and inconstant. Staining of freezing technique sections by Sudan III and Scarlet R has shown that these lipids take stain with less intensity than neutral fat which gives the corresponding test. They do not stain uniformly by Smith-Dietrich's technique for "lipines", some parts only of them give a positive reaction. Usually in each cell that contains these lipids, a fraction of them consists of "lipines" (chiefly lecithin), the remainder being lipids which do not give a positive Smith-Dietrich reaction. The lipids in question are now under more detailed investigation in connection with their appearance also in many other organs than ovary. So far, it can be said that they are a mixture of different lipids other than neutral fat in conjunction sometimes with a pigment.

*Adipose Tissue (Neutral Fat) in Mesovarium* (fig 1, VIII)—The amount of adipose tissue, the cells of which are filled mainly with neutral fat in the mesovarium, in animals on diet "N" (controls) is approximately on the same level throughout life.

In animals on diet "B" (vitamin E deficient) the adipose tissue is scarce, and disappears completely after about 380 days of the diet.

On diet "K" (vitamin E rich) the amount of adipose tissue quickly rises, and is soon—after 50 days of the diet—three times greater than in control animals. This level is maintained until the 480th day of the diet, and then it rises suddenly again to reach, after 590 days of the diet, an amount surpassing the amount in a control animal roughly nine times. In addition, beginning at 460 days of diet "K", cells filled with neutral fat appear in the ovarian tissue along the vessels and in corpora lutea.

On diets "W" and "E" the adipose tissue promptly disappears.

#### ANALYSIS OF RESULTS

Summarising the above results, it can be said that the changes concern (1) elements of germinal epithelium origin (primordial ova, follicles and interfollicular tissue), and (2) quantitative and qualitative disposition of lipids in the ovary and its neighbourhood (mesovarium). On diets "B" and "W" (vitamin E deficient) elements of germinal epithelium origin were decreased, and on diets "K" and "E" (vitamin E rich) were increased. In animals kept on vitamin E deficient diet neutral fat disappeared from the mesovarium, and lipids other than neutral fat made their appearance in ovarian tissue, and on vitamin E rich diet the amount of neutral fat was markedly augmented.

From a generalised statistical analysis it can be found that the experimental animals show (a) only very slight changes during the first six weeks of experimental diets, and (b) moderate, but obvious, changes during the first year of vitamin E deficient and vitamin E

*Diets "B" and "K"* —The ovaries of twenty-six vitamin E deficient (diet "B") animals are relatively large, hard, and yellowish in macroscopic examination. Their histological pictures (Pl II, figs 9 and 10) show grave abnormalities: very few corpora lutea and follicles, primordial ova and interfollicular tissue very scarce or completely absent. The medulla, filled copiously with cells containing lipids other than neutral fat, has invaded the cortical part of the ovary, and in some places reaches the surface of the organ. In the final stage of vitamin E deficiency the ovaries have no resemblance at all to a normal ovarian picture: under flattened germinal epithelium, the whole organ is a mass of cells well filled with lipids other than neutral fat, in that mass few clusters of connective tissue cells can be found, and there are few islets of cells resembling follicular cells but not establishing any regular follicles and without ova, primordial ova, follicles, and corpora lutea are lacking, occasionally one can find some single cells resembling cells of interfollicular tissue. The mesovarium contains no adipose tissue whatever, and consists of fibrous connective tissue and plain muscle fibres.

In contrast with the ovaries of animals on diet "B", the female gonads of fourteen vitamin E rich (diet "K") animals are smaller than in controls, yet their histological picture resembles that of a normal ovary with the chief differences: the interfollicular tissue is conspicuously more abundant, more normal and degenerating follicles are seen (Pl II, fig 11), some degenerating follicles contain blood, and a few adipose tissue cells, filled with neutral fat, can be found along the vessels. There are no lipids<sup>1</sup> which are so prominent in vitamin E deficient animals and which, in very small amount, make their appearance in control animals. In the mesovarium and subperitoneally in the neighbourhood of the ovary, the amount of adipose tissue is so enormous that there was in a few cases some difficulty during autopsy in finding the ovary itself in the fatty tissue.

In addition to the macroscopic and microscopic changes in the ovaries, there is a striking difference in the general appearance of mice on diets "B", "K", and "N" in regard to the amount of subcutaneous and subperitoneal fat. Animals on diet "B" (vitamin E deficient) show a pronounced leanness, animals on diet "K" (vitamin E rich) show a marked obesity, the diet "N" (stock) animals occupying a middle place (Pl II, fig 12).

#### DISCUSSION AND GENERAL CONCLUSIONS

*Previous Findings* —A histological study of ovarian structure in mice in relation to vitamin E not being available, the results that have been obtained can be compared only with the descriptions of rat ovaries

<sup>1</sup> In two animals, however, a few scattered cells, with minute amount of lipids other than neutral fat, had been found.

(b) *Animals 12 to 55 Weeks old kept 9 to 52 Weeks on  
Appropriate Diet*

*Diet "N"*—The ovaries of these fourteen control animals are normal mature organs (Pl I, fig 6) with few corpora lutea, all about the same stage as they all originate from the last oestrus or at most from the two last periods, and with a small number of primary follicles and primordial ova

*Diets "B" and "K"*—The ovaries of twelve animals on diet "B" contain a great number of corpora lutea which take the stain in different degrees, an indication, according to Allen [1922], that they are of various ages and originate from several periods of oestrus. Follicles are scarce and small, with few follicular cells, but they are usually maturing. There are fewer primary follicles and primordial ova than in controls, and much fewer than in animals on diet "K". Cords of interfollicular tissue are scarce and consist of a row of single cells. The medullary part of the ovary gives off branches which wedge in between corpora lutea. In the medulla, near the hilum, and between corpora lutea, numerous cells forming disseminated agglomerations can be seen, most probably of connective tissue origin and macrophages, filled with lipids other than neutral fat. In the mesovarium the amount of adipose tissue, consisting almost entirely of neutral fat, is less than half of its amount in controls.

The ovaries of ten animals on diet "K" show few corpora lutea, usually of last oestrus origin. Although the number of follicles is approximately equal in this and control diets, the follicles in diet "K" consist of more layers of follicular cells in comparison with diets "N" and "B", and more of them undergo an atretic involution. There are many more primordial ova than in the controls, and numerous mitoses are observed in the germinal epithelium. The interfollicular tissue forms a considerable part of the ovarian structure in the form of thick multi-layered cords and of spherical or irregular clusters and agglomerations (Pl I, fig 7). In the mesovarium, adipose tissue is much more abundant than in control animals.

(c) *Animals 56 to 116 Weeks old kept 53 to 113 Weeks (over  
One Year) on Appropriate Diet*

*Diet "N"*—The ovaries of these nineteen control animals show all elements of ovarian structure adequately developed (Pl II, fig 8), although the mice are approaching old age. There are only fewer primordial ova, fewer developing follicles, and less interfollicular tissue than in younger animals, the diminution in structures of epithelial origin is explained, however, by the age of the animals. Also a very small number of cells, filled with lipids other than neutral fat, appear in the medullary part of the ovary.

A more or less generalised conclusion may be drawn that vitamin E has some connection with the normal structure and development of germinal epithelium

*Lipids*—Bisceglie [1928] had noted some increase of "lipoids" in the rat ovary during vitamin E deficiency, but he did not attribute much importance to this observation since he did not mention it in the general conclusions and summary of his publication

In the analysis of the results of the present research, the changes in the amount of lipids in the mouse ovary are the most conspicuous phenomenon

During vitamin E rich diet no other lipids than neutral fat appear in the ovary and, moreover, the adipose tissue, containing neutral fat, is very abundant in the mesovarium and, as previously reported [Menschik, 1944], in subcutaneous and subperitoneal tissues. During vitamin E deficient diet, the adipose tissue disappears with a simultaneous appearance in ovarian tissue of lipids other than neutral fat. It should be remembered that both diets, vitamin E rich and vitamin E deficient, have been identical in all constituents except synthetic vitamin E. Under the experimental conditions of my investigations, vitamin E promotes the production of neutral fat in an abnormal quantity, and lack of vitamin E induces the appearance in the ovaries of a mixture of lipids other than neutral fat. These lipids—although connected often with a pigment, recently observed in rats during vitamin E deficiency by Mason and his co-workers in ovaries, muscles, and adipose tissue [Mason and Emmel, 1944, 1945, Mason, Dam, and Granados, 1946]—are composed mainly of unsaturated fatty acids, phospholipids, and cholesterol, as preliminary investigations [Menschik, 1946] had shown.

A general conclusion may be suggested that vitamin E is connected in some manner with lipid metabolism.

*Physiological Function*—The physiological function of vitamin E is still obscure.

Mason [1933], being first to discuss the physiological rôle of vitamin E, suggested that the vitamin is necessary for normal synthesis of nuclear chromatin. John [1939] attributed to vitamin E a rôle in the oxidation-reduction mechanism of cells. Adamstone [1934] abandoned his own idea of a rôle in cell-proliferation and replaced it by the hypothesis that the vitamin is related to the metabolism of anthracene compounds [Adamstone, 1941]. Shute's [1936] idea of equilibrium between vitamin E and oestrogens has been criticised [Cuthbertson and Drummond, 1939].

A connection of vitamin E not only with steroids but other lipids also has been suggested. Ever since Morgulis and Spencer [1936] found a disturbance in the amount not only of cholesterol but also of total lipids, an interrelation of lipids and vitamin E has been voted by an

during vitamin E deficiency recorded by many authors. Histological sections, however, have usually not been serial, wheat-germ oil has been used as the source of vitamin E instead of a synthetic substance, and the duration of the experiments has not exceeded 360 days. Most of such investigations have given negative results.

Martin and Moore [1939] found ovarian cysts during vitamin E deficiency.

Bisceglie [1928] describes, in a few rats (10 experimental animals and 10 controls), changes in the ovaries during vitamin E deficiency, serial sections having been made. He found marked changes—only after 170 days of vitamin E deficiency, and that only in one animal—concerning especially the follicles with complete degeneration of germinal epithelium and with an increase of “lipoid” substances in interstitial cells. Control animals receiving the same avitaminous basic diet, supplemented with wheat-germ oil, retained a normal ovarian structure till the end of his experiment.

Muller [1939] found in rats that during E-avitaminosis the ovarian cycle is retained for a long time, and there are no structural changes in the gonads. Nevertheless, after longer duration of avitaminosis—not sooner than during the 6th month—he observed in some animals conspicuous changes in the ovaries, the whole organ being smaller at the expense of follicle-producing tissue. He found fewer maturing follicles, and follicular cells showing regressive changes. In the final stage, in the ovary, reduced to one-tenth of the normal mean weight, not a single normal mature follicle could be seen, only a few single, old corpora lutea were present, and the small-celled interstitial tissue was especially abundant.

Recently Mason and Emmel [1944] observed during vitamin E deficiency, in the ovaries of rats, a gradual accumulation of pigment which they regarded as an abnormal product, probably an unusually stable protein-lipid combination, liberated in an otherwise normal process of cell degeneration in atretic follicles and regressing corpora lutea.

*Germinal Epithelium*—The results of my experiments on mice show that primordial ova, primary follicles, the total amount of follicular cells and interfollicular tissue—all elements of germinal epithelium origin—were increased by administration of vitamin E, and decreased in vitamin E deficiency.

Although Bisceglie and Muller have obtained results with rats different, in some respects, from my results with mice, the positive results of both researches, nevertheless, are in accord in showing that in vitamin E deficiency the germinal epithelium of the ovary is handicapped. This is to some extent analogous with the changes observed by many authors in the rat testis [Evans and Burr, 1927, Mason, 1940, and others], although not in mice [Goettsch, 1942].

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ever-increasing number of authors [Morgulis *et al*, 1938, Kudryashov, 1942, Houchin and Mattill, 1942, Gottlieb, Quackenbush, and Steenbock, 1943, Heinrich and Mattill, 1943, Barnes *et al*, 1943, Dam, 1944 *a*, 1944 *b*] So far, the facts known about vitamin E function are far from being satisfactory [Mason, 1944, Hickman and Harris, 1946]

The obvious differences of ovarian structure, in mice on vitamin E deficient and vitamin E rich diets, described and illustrated in this paper seem to support, from a morphological point of view, the suggestion of a close functional relationship between vitamin E and lipids

### SUMMARY

A serial histological study has been made of the ovaries of mice fed from the day of weaning on several different diets, the principal being a vitamin E deficient diet and one containing alpha-tocopherol ("Ephynal" Roche) as the only vitamin E source. Control animals were kept on stock diet, lettuce being the main source of vitamin E. Animals were examined after periods ranging from 7 to 791 days of the particular diet.

1 Animals on vitamin E rich diet, compared with controls, show ovaries with more primordial ova and primary follicles. follicular cells and interfollicular tissue of epithelial origin are more abundant, but degenerating follicles are numerous. there are fewer mature follicles and corpora lutea, both of which are enlarged. After prolonged vitamin E rich diet the ovary is small with degenerating follicles containing blood. neutral fat is present in the ovarian medulla and adipose tissue is extremely abundant in the mesovarium.

2 Animals on vitamin E deficient diet, compared with controls, show ovaries with fewer primordial ova and less interfollicular tissue. follicles contain fewer follicular cells. corpora lutea are increased, but are smaller and of various ages. medullary lipids, not readily soluble in fat solvents, are prominent, yet there is complete absence of neutral fat in both ovary and mesovarium. As the vitamin E deficiency continues, the lipids invade the ovarian cortex. After prolonged vitamin E deficient diet the ovary, comparatively large, is hard and yellowish and contains only cells filled with lipids other than neutral fat.

The ovarian changes summarised under 1 and 2 appear to be quantitative rather than qualitative, but it seems certain that vitamin E stimulates the development of all elements of germinal epithelium origin, and that it influences fat metabolism in the ovary and its neighbourhood so that neutral fat increases, while the formation of other lipids is inhibited.



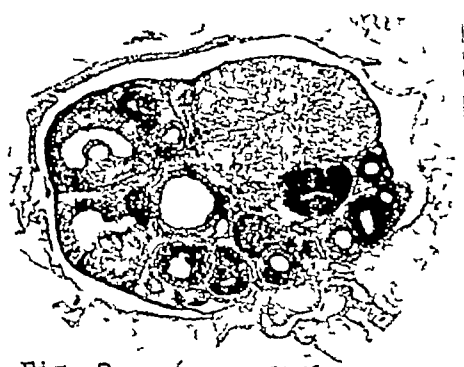


Fig. 2.

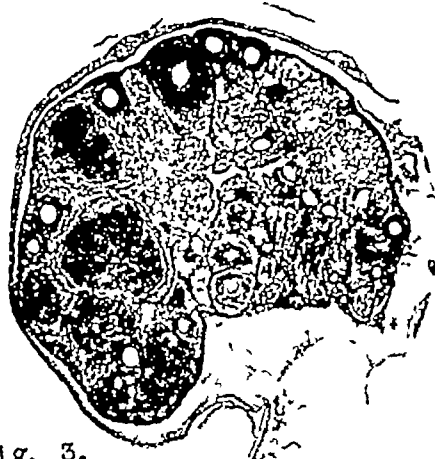


Fig. 3.

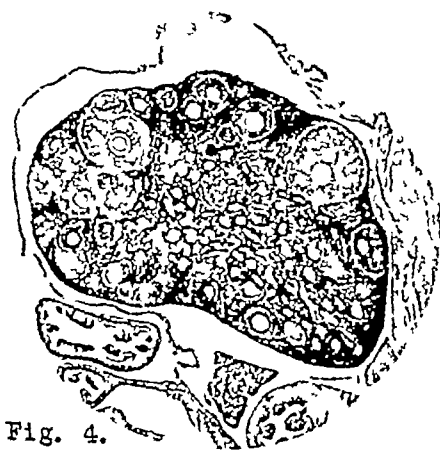


Fig. 4.



Fig. 5.

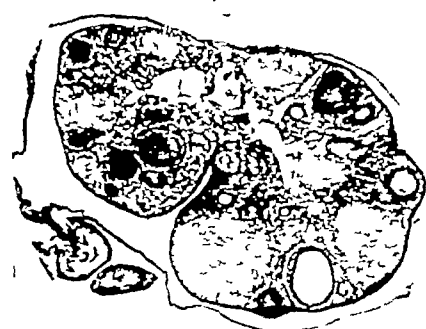


Fig. 6.

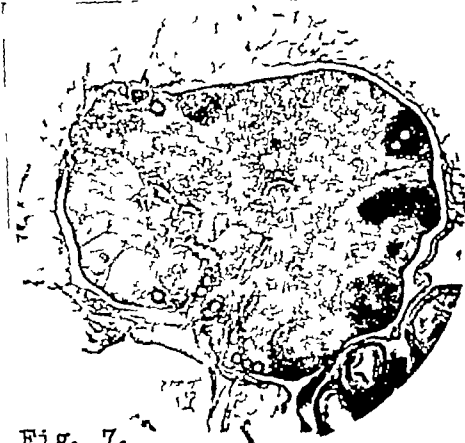


Fig. 7.

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#### EXPLANATION OF FIGURES

FIG 1—Diagrammatic representation of different parts of ovarian structure during control and experimental diets

- I The number of corpora lutea in one ovary
- II The mean percentage of the surface of ovarian sections occupied by follicular cells.
- III The mean number of primary follicles in one ovarian section
- IV The percentage of follicles undergoing degenerating changes
- V The mean percentage of the surface of ovarian sections occupied by interfollicular tissue
- VI The mean number of primordial ova in one ovarian section
- VII. The mean percentage of the surface of ovarian sections occupied by cells filled with lipids other than neutral fat
- VIII. The mean proportion of sectional area occupied by periovarian adipose tissue to sectional area of ovary

PLATE I

- FIG 2—Photomicrograph of an ovarian section of a mouse Erhlich's hæmatoxylin and eosin Diet "N" (control) Age 58 days  $\times 40$   
 FIG 3—The same Diet "K" (vitamin E rich) 35 days Age 58 days  $\times 45$   
 FIG 4—The same Diet "W" (vitamin E and other vitamins deficient) 29 days Age 64 days  $\times 48$   
 FIG 5—The same Diet "E" (vitamin E rich, and other vitamins deficient) 29 days Age 64 days  $\times 62$   
 FIG 6—The same Diet "N" (control) Age 86 days  $\times 27$   
 FIG 7—The same Diet "K" (vitamin E rich) 63 days Age 86 days  $\times 25$

PLATE II

- FIG 8—Photomicrograph of an ovarian section of a mouse Erhlich's hæmatoxylin and eosin Diet "N" (control) Age 461 days  $\times 28$   
 FIG 9—The same Diet "B" (vitamin E deficient) 369 days Age 404 days  $\times 30$   
 FIG 10—The same Diet "B" (vitamin E deficient) 438 days Age 461 days  $\times 27$   
 FIG 11—The same Diet "K" (vitamin E rich) 369 days Age 404 days  $\times 29$   
 FIG 12—Photograph of three mice, litter mate sisters, on diets "N" (control), "B" (vitamin E deficient) and "K" (vitamin E rich), after the abdomen has been opened to demonstrate the amount of subperitoneal adipose tissue Diet "B" or diet "K" 587 days Age 611 days

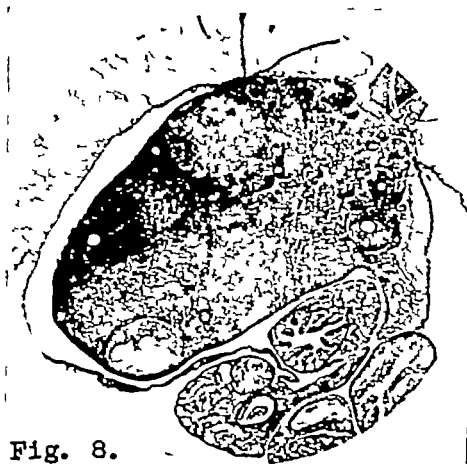


Fig. 8.



Fig. 9.

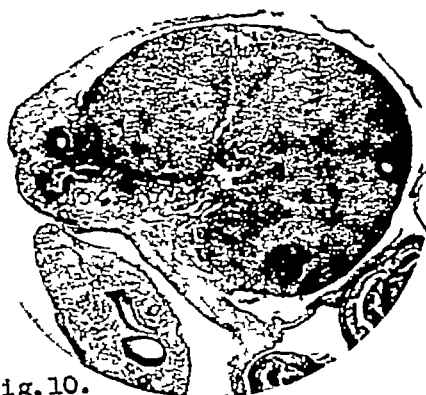


Fig. 10.

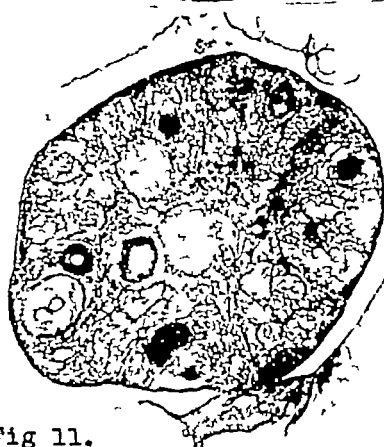


Fig. 11.

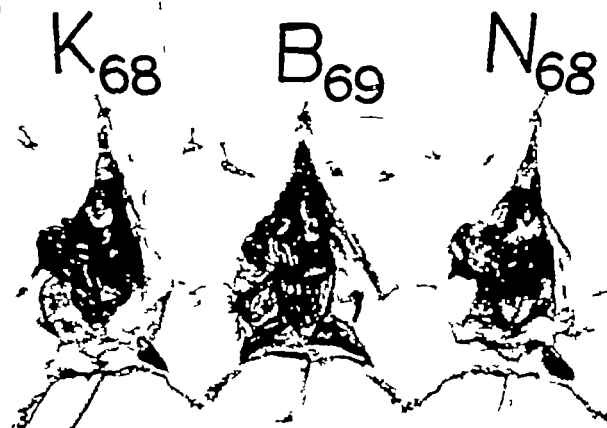


Fig. 12.

# THE RELATIONSHIP OF THE THYROID GLAND TO MUSCULAR WORK PERFORMED BY THE ALBINO RAT

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THE sluggishness of the hypothyroid patient, and the excitability and muscular tremor of the hyperthyroid patient are commonly observed [Joll, 1932, Hertzler, 1935] Whether these changes are due to the action of the thyroid hormone, or the lack of it, on the muscles themselves, or on the nervous system activating them has not been proven. It is difficult to measure the end point at which a human muscle is fatigued, as the subject of the experiment, unless possessed of unusual powers of endurance, will not continue to the point of exhaustion. Furthermore, a patient suffering from myxoedema is too lethargic and a patient with thyrotoxicosis is too easily distracted, for the experiment to be of value. Moreover, such a test would only measure an effect on the whole neuro-muscular mechanism concerned in the cortical control of movement.

This paper presents the results of a series of experiments designed to record the amount of work performed by the gastrocnemius muscle preparation in the anæsthetised rat, under conditions of normal thyroid metabolism, hyperthyroidism, and hypothyroidism. The experiment was designed, as far as possible, to imitate the normal movements of the rat hind-leg in running. Three groups of rats were used, normal, thyroidectomized, and normal rats with thyroid added to the diet.

## METHOD

Healthy white rats of both sexes, aged 4 months, were used for experiment. All rats were fed on a standard diet (meat-free) as follows: Bran 30, pollard 25, bone meal 15, pea meal 15, maize meal 15 (all gm per cent), wheat or kibbled maize *ad lib*.

The duration of each experiment was twenty hours, even if the muscle had meanwhile ceased to contract on stimulation. At the end of twenty hours the rat was killed, the muscle length and weight determined, and neck, thorax, and abdomen of the rat examined. In animals on which thyroidectomy had been performed, serial sections of the tracheal region were cut, to discover whether thyroidectomy





Diagram of the tracing of a 155 Gm thyroidectomised, iodine-fed rat. Period of sustained contraction, A-B, lasting 12 hours. Total tracing - 20 hrs.

FIG 1

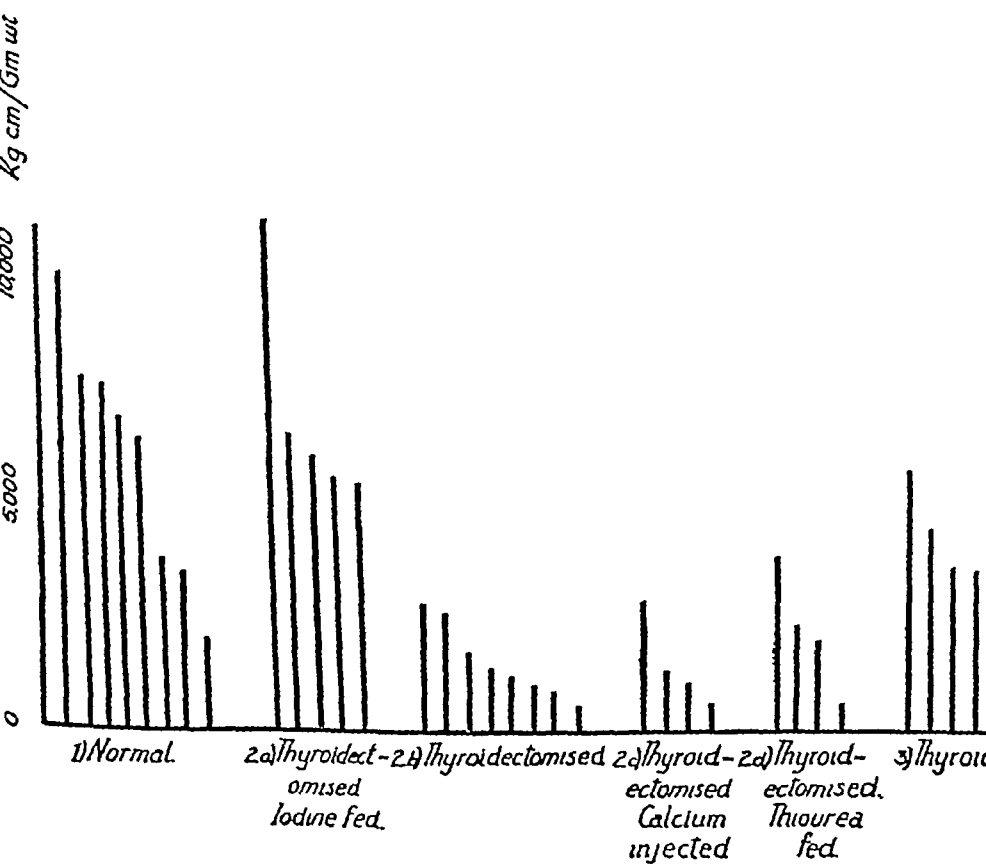


FIG 2

had been complete or not. In only a few cases one or two acini containing colloid were found in the angle between the trachea and the œsophagus. (To ensure complete thyroidectomy some operations were performed under direct vision with the dissecting microscope, while most of the remainder were inspected with this instrument at the close of the operation.)

The anæsthetic used was barbitone soluble, 35 mgm per 100 gm body-weight of rat, given by intraperitoneal injection. When the experiment had been in progress about eight hours, a further 5-10 mgm were given subcutaneously. The size of this dose depended on the weight of the rat and the depth of anæsthesia at this stage. In this way an approximately even depth of anæsthesia was maintained throughout the experiment. It was difficult to maintain anæsthesia for more than twenty hours, and it was felt that results could not be relied on to be physiologically significant after that time.

The insertion of the tendon of the gastrocnemius muscle was exposed, and the tarsal bone distal to the insertion divided. The tendon was then attached to the isometric lever by a steel wire. A tension of approximately 70 gm wt was maintained on the muscle during relaxation. The muscle belly was not exposed. An incision was made in the thigh, the muscles separated at the lateral intermuscular septum, and the sciatic nerve exposed. The stimulating electrodes were placed in contact with the nerve, which was crushed proximal to the electrodes, and the anterior tibial branch was cut distal to the electrodes. The skin incision was closed around the electrodes by a stitch.

The stimuli consisted of brief tetani, 2 every second, with an interval between tetani, approximately twice the duration of the tetanus, i.e. tetani of about 0.15 sec with intervals of 0.35 sec. This ensured adequate blood supply to the muscle in the intervals between contractions. The stimulating apparatus produced shocks at the rate of 50 per second, which were maximal for the motor nerve to the muscle. In every case, when twenty hours had elapsed, pin electrodes were inserted into the muscle, and the same cycle of stimulation observed. In all cases save two, no increase in the height of contractions was observed. Slightly increased contractions for less than one minute were observed in the two exceptions.

The tension developed by the muscle was recorded isometrically, the tracing being made optically, on a slowly moving roll of photographic paper. Each contraction of the muscle produced a vertical line on the paper, varying in height in direct proportion to the tension developed. Since the paper moved at a slow uniform speed this produced a zone of darkened paper, the area of which was directly proportional to the work performed. After developing, the area was measured by means of a planimeter.



TABLE

Group	No of Animals	Mean Body Weight (gm)	Mean Work Kg cm / Gm Muscle	Standard Deviation	Time (Hr)	Remarks
1 Normal, Controls	9	146	6589	2811	20	Unfatigued No sustained contraction
2 (a) Thyroidectomized 6 weeks previously Iodine added to drinking water (approximately 2-4 microgram intake daily)	5	176	7095	2434	20	Unfatigued Sustained contraction in early stage
2 (b) Thyroidectomized 6 weeks previously Plain drinking water	8	128	1382	888	20	Unfatigued Sustained contraction in early stage
2 (c) Thyroidectomized 6 weeks previously Plain drinking water 0.1 gm calcium gluconate injected one hour before experiment	4	139	1439	1131	20	Unfatigued Sustained contraction in early stage
2 (d) Thyroidectomized 3 weeks previously Thiourea added to drinking water for 14 days (approximately 15-25 milligram daily)	4	179	2252	1406	20	Unfatigued Sustained contraction in early stage
3 Thyroid fed normal rats, 64 milligram daily for 2 weeks	7	141	3592	1326	20	Unfatigued No sustained contraction

The work done was calculated as follows Work done in one contraction of muscle (gm cm) = Tension developed (gm)  $\times \frac{1}{3}$  muscle length (cm) Thus the total work per gm wt of muscle for the whole experiment is given by the formula

$$\text{Work (gm cm)} = \frac{120 t a C}{6 L g}$$

where

$a$  = area of tracing in sq cm

$L$  = length of tracing in cm

$C$  = length of muscle in cm

$g$  = weight of muscle in gm

$t$  = time of experiment in min

120 = tetani per minute

## RESULTS

The results are summarised in the graph of fig 2 and in the accompanying table In the case of animals of Group 2 (thyroidectomized), on commencement of stimulation after contracting normally for a few minutes the muscle entered a phase of sustained contraction The isometric lever became stationary in the contracted position and remained there for a period varying from one quarter to two hours This phase ended by the muscle beginning to relax slightly, with subsequent contraction at each stimulus This degree of relaxation between stimuli increased, until the muscle was contracting and relaxing normally at each stimulus If the stimulating apparatus was stopped during the phase of sustained contraction, the muscle relaxed within one or two seconds, but the sustained contraction recurred on recommencing the stimulation Once this phase of sustained contraction had passed, it did not recur It was not observed in Groups 1 and 3 Further, in some animals additional to those whose results are recorded, stimuli were applied by pin electrodes thrust through the muscle during the phase of sustained contraction This had no effect on the sustained contraction, which continued, and then passed off in the usual fashion It is possible then that changes in the nerve or neuro-muscular junction were not responsible for this phase

Acting in the belief that this phase might be an effect of hypocalcaemia due to parathyroidectomy, a sub-group, 2c, of animals was given calcium in the form of calcium gluconate, 1 ml of a 10 per cent aqueous solution (equal to 8.9 mgm calcium) by intraperitoneal injection, one hour before the commencement of the experiment This quantity of calcium, if distributed evenly throughout the body tissues would raise the blood calcium level on an average by 0.4 mgm / 100 ml It is likely, however, that as calcium gluconate produces an effect 15-20 minutes after injection, and this effect lasts 6-8 hours

apart from Group 2 *a*, thyroid deficiency diminished considerably the ability of rat muscle to perform work

When thyroid gland substance is fed to normal animals work is again performed at a lesser rate than normally. The probability of the difference observed between Groups 1 and 3 occurring by chance alone is between 1 in 50 and 1 in 20. In this group there was no phase of sustained contraction. It therefore seemed probable that the causative factor of the sustained contraction phase was thyroid deficiency. If that is so, treatment of thyroidectomized animals by replacement doses of thyroxine should abolish the phase of sustained contraction. The sustained contraction has not been especially investigated.

Much larger deficiencies in work performances were observed by Malcolm and Whitehead [1944] in thyroidectomized rats. This may be attributed to the different conditions under which their experiments were performed.

(1) Their investigations recorded the work performed by muscle twitches, not brief tetani.

(2) Their method of recording was isotonic, so that no work would be recorded when the muscle contraction fell below a limiting value. With the present isometric method even the smallest contractions contribute to the aggregate work. Thus the present method gives a more acceptable value for the total performance of work by a muscle.

(3) With the onset of the phase of sustained contraction, no work would be recorded for an hour or more, and their experiments were not continued sufficiently long to discover the later recovery with restored relaxation.

When allowance is made for these different conditions, there is no serious discrepancy in the two sets of observations. In addition, Malcolm and Whitehead showed that treatment of thyroidectomized animals by replacement doses of thyroxine approximately restored to normal the work output of muscles, an experiment not attempted with the present technique.

Thus in the white rat, it is shown that the gastrocnemius preparation will for many hours perform work at a rate varying with the thyroid metabolism.

A normal rat performed more work than an animal which is either hypothyroid or hyperthyroid. No animal, hypothyroid or hyperthyroid, was able to be fatigued within the duration of the experiment—twenty hours. However, all thyroidectomized animals showed an early phase of sustained contraction which may be mistaken for fatigue. Since each animal, apart from the period of sustained contraction, performed work at its own uniform rate, significant difference in work performance should be obtained in experiments lasting six or eight hours.

[Shelling, 1935], that the blood calcium would be raised above normal during the first half of the experiment, then falling to its previous value. As the time courses of the rates of work were similar, and there was no significant difference between the means of sub-groups 2 *b* and 2 *c*, it is probable that variations in the calcium level were without effect (It should be mentioned that many animals suffered from tetany for two or three days after thyroidectomy, but appeared to recover when calcium was added to the drinking water.)

Apart from this initial phase of sustained contraction, animals in each group performed work at a rate approximately uniform with others in the same group. Those in Groups 1 and 2 *a* continued to work at a high rate, while 2 *b*, 2 *c*, 2 *d*, and 3 worked at a low rate.

An attempt was made to feed rats on thyroid for more than two weeks. This experiment failed because these rats succumbed to the anaesthetic after an average of  $3\frac{1}{2}$  hours.

The depth of anaesthesia has an important influence on the performance of muscular work. Deep anaesthesia greatly diminished the performance of work, while if the anaesthesia became too light, the animal spoilt the experiment by its spontaneous movements.

A few animals performed the whole experiment with pin electrodes thrust through the muscle instead of sciatic nerve stimulation. The response of these animals pursued similar courses to those whose results are recorded. It was felt, however, that this method of stimulation caused unnecessary trauma to the muscle.

## DISCUSSION

Statistically there is no significant difference between the work performed by Groups 1 and 2 *a*, but these groups differ significantly from the remaining thyroidectomized animal experiments, sub-groups 2 *b*, *c*, and *d*. The probabilities that these differences are due to chance alone are less than 1 in 100 for 2 *b* and 2 *c*, and between 1 in 50 and 1 in 100 for 2 *d*. (Calculations were made on the formula for the difference between two means, using the *t* Table [Fisher].)

No explanation can be offered for the surprisingly large work output of Group 2 *a*. Though the iodine added to the drinking water would give adequate thyroxine production in the presence of thyroid, it would cause negligible production in Group 2 *a*, for the extra-thyroidal synthesis of thyroxine from iodine has recently been shown to be exceedingly inefficient [Morton *et al*], only a minute fraction of the administered iodine being so converted [Purves and Greisbach]. Moreover, despite the iodine, the sustained contraction is observed in Group 2 *a* in common with the other thyroidectomized animals (Groups 2 *b*, *c*, and *d*). It is evident that further investigation is needed to throw light on these apparently conflicting findings. However, it seems that

# DETERMINATION OF BLOOD VOLUME IN DOG BY MEANS OF VISUALLY LABELLED ERYTHROCYTES By A NIZET<sup>1</sup>

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(Received for publication 23rd April 1947)

THE use of labelled erythrocytes usually prepared by means of radioactive or heavy isotopes has already added much to our knowledge of the blood under physiological and pathological conditions [Hevesy, 1940]. Studies in this laboratory on the structure of the bone marrow [Nizet, 1943 *b*, 1947] have been undertaken using visually labelled erythrocytes produced by the action of phenylhydrazine on red blood corpuscles. Further, Lambrechts and Nizet [1943] have shown that erythrocytes labelled in this way can be used for blood-volume determination. Erythrocytes treated with phenylhydrazine contain Heinz granules [Heinz, 1890, Moeschlin, 1941], and these granular erythrocytes are easily identified in stained smears observed under dark ground illumination [Nizet, 1943 *a*, 1944]. The method of Lambrechts and Nizet [1943] for blood-volume determination involved the transfusion of blood containing labelled erythrocytes from an animal which had received an intravenous injection of phenylhydrazine, into another animal, the blood volume of which it was desired to measure. The method has been improved and simplified by preparing the labelled erythrocytes *in vitro*.

## *Preparation of Visually Labelled Erythrocytes by Phenylhydrazine*

In the first place, it is necessary to discover the optimum concentration of phenylhydrazine which, when added to a blood sample, will produce Heinz granules in 90–100 per cent of the erythrocytes without leaving an excess of phenylhydrazine. Since phenylhydrazine reacts only with oxyhaemoglobin and has no effect on reduced haemoglobin [Nizet, 1946 *a*, *b*], it is also important to saturate the blood samples with oxygen before the addition of phenylhydrazine.

The following series of experiments carried out on human blood

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## SUMMARY

1 Muscular work performed by the sciatic nerve-gastrocnemius muscle preparation in the anaesthetised rat is recorded when normal, high, and low thyroid hormone levels are present

2 Hypothyroid animals performed less work than normal animals

3 Hyperthyroid animals performed less work than normal animals

4 Thyroidectomized animals, with iodine added to the diet, performed work at a rate not significantly different from normal animals

5 All animals on which thyroidectomy had been performed, showed a phase of sustained contraction, lasting  $\frac{1}{4}$  to 2 hours, at the commencement of the experiment This phase was not due to hypocalcaemia, and possibly was not due to defective neuro-muscular transmission

6 No animal, normal or abnormal in thyroid metabolism under the conditions stated, showed muscular fatigue during the twenty hours of the experiment

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100, 50, 25, 12.5, 6.25 and 3.125 mg per cent, giving finally 100, 50, 25, 12.5, 6.25, 3.12, 1.56 and 0.78 mg per cent. Thirty-five minutes later the changes in the oxyhaemoglobin were noted, the bloods were then centrifuged and the supernatant plasmas put aside. The packed red cells were stained by brilliant cresyl blue in our usual manner, and the percentage of erythrocytes with Heinz granules established for each concentration. Two experiments are quoted in Table II. The plasmas of experiments 1 and 2 (Table II) were then mixed with an

TABLE II

Expt	Phenylhydrazine mg per cent	100	50	25	12.5	6.25	3.125	1.56
1	Colour of the blood	B	B	B	B	R	R	R
	Percentage of erythrocytes containing Heinz granules	100	100	98	10	0	0	0
2	Colour of the blood	B	B	B	RB	R	R	R
	Percentage of erythrocytes containing Heinz granules	100	99	70	5	0	0	0

B = brown, R = red

equal volume of the same fresh blood. It was found that the plasmas removed from the lowest phenylhydrazine concentrations giving complete change of colour and 95–100 per cent of granular red cells (10–25 mg per cent for the human blood) did not produce any alteration in fresh erythrocytes after contact for 35 minutes. These results show that if there is an excess of phenylhydrazine present, it is insufficient to produce a second reaction.

#### *Determination of Blood Volume of Dog*

The optimum concentration of phenylhydrazine to produce 90–100 per cent of granular cells in dog's blood, determined in a similar manner has been found to be from 50 to 100 mg per cent. The addition of phenylhydrazine to the blood causes frothing, which is dispersed by one drop of a mixture containing 10 per cent octyl alcohol and 90 per cent ethyl alcohol. The percentage of granular red cells is determined in this blood sample as well as in the blood of the recipient dog before the injection. A known volume (10 c.c. to 50 c.c.) of the labelled blood is then injected intravenously into the dog and a blood sample taken 5 to 40 minutes after the injection and examined for granular cells.

demonstrates that the reaction with oxyhæmoglobin of the red blood cells depends upon the concentration of phenylhydrazine

Equal samples of the same citrated blood were mixed with a constant volume of phenylhydrazine solutions of decreasing concentrations. The phenylhydrazine solutions were prepared by dissolving it in an equal volume of methyl or ethyl alcohol, diluted afterwards by NaCl 0.9 per cent

*Experiment 1* — 10 c.c. of phenylhydrazine (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.2 mg per cent) were mixed with 0.2 c.c. of blood

*Experiment 2* — 4 c.c. of phenylhydrazine (125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.95, 0.98, 0.49 and 0.25 mg per cent) were mixed with 1 c.c. of blood

*Experiment 3* — 1 c.c. of phenylhydrazine (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 and 0.39 mg per cent) were mixed with 1 c.c. of blood

The final concentrations of phenylhydrazine were respectively the same in the three experiments (100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.39 and 0.2 mg per cent). The absolute amount in the first experiment for the same volume of blood, was 50 times, and in the second experiment  $2\frac{1}{2}$  times that of the third experiment.

After contact of 35 minutes ( $\pm 20^\circ \text{C}$ ) the colour of blood turned from red to brown in the three experiments for the phenylhydrazine concentrations higher than 3 mg per cent. For the lower concentrations the oxyhæmoglobin remained unchanged. The results are summarised in Table I.

TABLE I

Expt	Phenylhydrazine mg per cent			100	50	25	12.5	6.25	3.12	1.56	0.78	0.39	0.2
	c.c. sol	c.c. blood											
1	10	+	0.2	B	B	B	B	BR	R	R	R	R	R
2	4	+	1	B	B	B	B	BR	R	R	R	R	R
3	1	+	1	B	B	B	B	BR	R	R	R	R	R

B = brown, R = red

That the Heinz granules appear simultaneously with the alterations in hæmoglobin has been demonstrated in the following manner.

Ten different blood samples (1.5 c.c.) were mixed with 0.5 c.c. phenylhydrazine solution in decreasing concentrations (400, 200,



TABLE III

Series 1 (Labelled (L) blood from a different dog)

Expt	Wt of recipient, kg	Vol of L blood injected (diluted), c c	Erythrocytes per mm. <sup>3</sup>		Granular red cells, per cent				Total blood volume, c c	Blood vol/kg body-wt c c
			L blood (diluted)	Recipient	Labelled blood	Recipient				
						Before	7 min	15 min		
1	6.7	25	4,430,000	7,040,000	100	0	4.87		323	48
2	6.7	20	4,700,000	6,150,000	99.6	0		2.733	557	83
3	10.2	20	5,350,000	6,400,000	100	0		1.3	1284	79

Series 2 (Labelled (L) blood drawn from the dog under experiment)

Expt	Wt of animal, kg	Vol of L blood injected (diluted) c c	Dilution		Vol of injected blood (corrected) c c	Granular red cells, per cent				Total blood volume, c c	Blood vol/kg body wt c c
			Citrate	Phenylh		Labelled blood	Recipient				
							Before	5 min	35 min		
4	8.2	18.7	1/11	1/10	15.3	97	0	2.36		629	78
5	22.7	50	1/10	1/10	40.5	100	0	3.86	3.75	1065	47
6	7.8	30	1/10	1/10	24.3	100	0	5.76	5.69	424	55
7	5.8	27	1/10	1/5	19.4	100	0.16	6.26	6.57	302	52
8	21.1	50	1/10	1/5	36	100	0	2.6	2.77	1343	64
9	6.4	17.5	1/10	1/5	12.6	100	0	2.65	2.45	495	77
10	9.4	40	1/10	1/5	28.8	97	0	3.66	3.81	732	78

The blood volume  $V$  of the recipient dog is calculated by the following formula —

$$V = \frac{NvE}{ne} \quad (I)$$

$N$  = percentage of erythrocytes labelled in the injected blood,

$n$  = percentage of erythrocytes labelled in recipient's blood after the injection,

$v$  = volume of injected "labelled" blood,

$E$  = number of erythrocytes per  $\text{mm}^3$  in the injected blood,

$e$  = number of erythrocytes per  $\text{mm}^3$  in the recipient's blood

If the blood to be labelled is drawn from one animal and the blood volume determined on a different one, it is necessary to count the red corpuscles in the injected blood and in the recipient's blood. On the contrary, if the injected blood is obtained from the dog, the blood volume of which is to be determined, it is only necessary to know the dilution of the labelled blood by citrate and phenylhydrazine solutions. In this case where  $E=e$ , after correction of the injected volume, formula (I) may be written as follows —

$$V = \frac{Nv}{n} \quad (II)$$

The results of ten determinations in normal dogs are transcribed in Table III. In experiments 1 to 4, samples of blood were taken 7 to 15 minutes after the injection. In experiments 6 to 10, blood samples were taken 5 minutes and 35 minutes after the injection in each case.

The concentration of phenylhydrazine was 100 mg per cent in all the experiments. Each labelled red cell count was based on a total cell count of 7400. The average blood volume of the normal dog was found to be 66 l c c per kg body-weight. In our experiments the extreme values are 47 c c /kg and 83 c c /kg, according to experiments 5 to 10, the results are the same if the blood is taken either 5 or 35 minutes after the injection, the mean difference is only 4 per cent. The results indicate that there is no noticeable destruction or elimination of the labelled elements during the first 35 minutes, and that the injected elements seem to be perfectly mixed with the circulating blood elements after 5 minutes.

We emphasise the fact that the blood volumes are calculated on the supposition that the ratio  $\frac{\text{plasma}}{\text{red cells}}$  is the same in all the vascular areas in which blood is circulating.

121111

# HÆMOGLOBIN CONCENTRATION, HÆMATOCRIT VALUE, AND SEDIMENTATION RATE OF HORSE BLOOD

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(Received for publication 25th September 1947)

THE experiments reported here were carried out as a preliminary to the study of the respiratory function of horse blood. The high sedimentation rate of horse blood necessitates certain modifications in technique for accurate comparison with the blood of other animal species. The viscosity and the concentration of r b c are the factors influencing the velocity of exchange of the respiratory gases.

## METHODS

Heparinised blood (1000 units of B D H heparin per 100 c c of blood) of 7 healthy horses were examined. For one experiment defibrinated blood was used. The determinations usually started 1-2 hours after the blood was collected from the jugular vein.

*Hæmatocrit* values were obtained by centrifuging the blood for one hour in capillary tubes 10 cm long at 3000 r p m or in shorter tubes (6 cm) for 15 min at 10,000 r p m.

*Hæmoglobin* was estimated as cyanmethæmoglobin [Stadie's method, 1920, modified by Wu, 1922] in the Hilger Spekker photo-electric absorptiometer using green (N5) filter. Hæmatocrit and hæmoglobin values were corrected for dilution of blood by heparin.

*Sedimentation rate* was measured in 200-mm long Westergreen tubes. A complete curve of sedimentation was obtained, the relative velocity curve being calculated from it in p c of the total length of the plasma column given by hæmatocrit value. Maximum velocities in mm/min were calculated from relative velocity curves.

*Viscosity* (plasma, serum, and blood) was determined as apparent viscosity in relation to water in an apparatus which was designed to avoid the influence of corpuscular sedimentation during the determination. The apparatus consisted of a container of about 20 ml capacity into which a capillary tube (Veridia) was introduced as shown in fig 1. The length of the capillary tube between two marks was 55 cm and it was kept in the horizontal position. The inner diameter of the capillary was 0.5 mm. One end of it was immersed in blood (5 ml) placed in the container, the other one was connected to an arrangement producing a

<sup>1</sup> In receipt of personal grant from the Agricultural Research Council

## SUMMARY

A new technique is described for the measurement of circulating blood volume, using visually labelled erythrocytes. These elements are prepared by the action *in vitro* of phenylhydrazine on the erythrocytes. The granular elements remain in the circulating blood long enough to allow good mixing. Successive determinations in the same animal show good agreement. The average circulating blood volume of the normal dog is found to be 66 c c per kg body-weight.

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TABLE I—V P O R B C, SEDIMENTATION RATE, HÆMOGLOBIN CONCENTRATION, AND VISCOSITY OF HORSE BLOOD

Expt No	Horse No	V P O r b c	Hb g p c	Hb g p c / V p c r b c	Relative viscosity of plasma at 38° C	Relative viscosity of blood at 38° C	Maximum rate of r b c sedimentation, mm /min	Remarks
1	1	27.60	9.51	0.3446		6.83	5.8	Excited
3	2	48.40	18.20	0.3600		4.64	0.6	
5	2	36.60	13.06	0.3568	1.58	4.53	3.0	
6	3	36.60	12.75	0.3480	1.67	4.26	4.0	
7	3	33.80	11.80	0.3490	1.65	4.00	7.7	
11	4	31.15	10.70	0.3430	1.85			
14	5	50.40	18.70	0.3710			5.9	
150	6	35.00	12.32	0.3520		4.73	5.9	At rest
152	7	34.70	12.32	0.3550	2.13	6.25	1.6	After exercise
152	7	43.87	16.75	0.3590	2.26	4.45		At rest
154	7	34.12	12.32	0.3611	2.05			After exercise
154	7	39.91	14.77	0.3701		4.39		At rest
156	7	34.31	11.94	0.3480	1.95			After exercise
161	7	36.82	13.17	0.3577		4.60		Prepared from blood ob- tained at rest
161	7	34.80				5.41		Prepared from blood ob- tained at rest
161	7	40.00				7.55		Prepared from blood ob- tained at rest
161	7	52.3						

constant negative pressure (40 mm Hg) at which the fluid was moving by suction through the capillary. The container was fixed into a rocking frame which was moving to and fro with equal rate for all determinations. The whole apparatus was placed in the thermostat. Time of movement of the column of fluid between two marks was measured with a stop watch (to 1/5 sec) and compared with the velocity of flow of water at the same conditions of temperature and pressure.

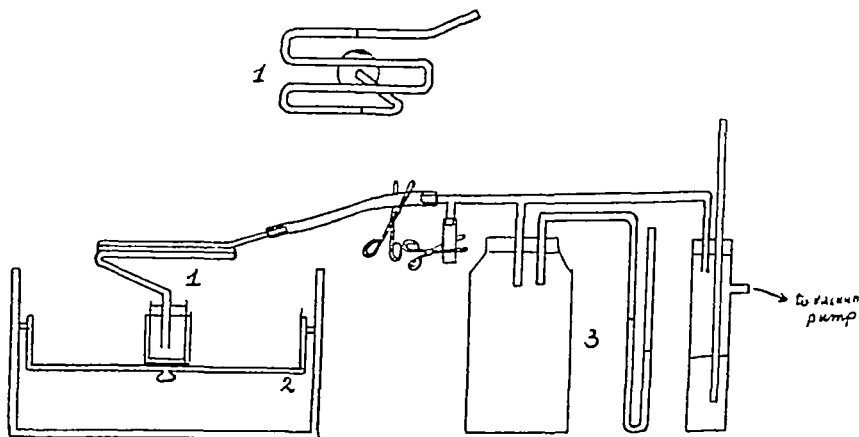


FIG 1 —Illustration of the viscometer (1) placed in a rocking frame (2) and connected to a constant pressure arrangement (3)

## RESULTS

The results are given in Table I

*Hæmoglobin Concentration, Hæmatocrit Value ( $V_{pcrb}$ ), and the  $Hb\ g\ pc/V_{pcrb}$  ratio*—The volume  $pc$  of  $rb$  varied from 27.6 to 50.4. Even in the same horse (No. 2) blood taken on two different days showed a marked difference in concentration of  $rb$ . In this case it was noticed that when for the first time the horse was bled it was much more excited than at the second bleeding. In this connection 3 experiments on horse No. 7 also showed a marked increase in volume  $pc$  of  $rb$  after short exercise (moderate trot about 10 min). These observations are in agreement with the results of Scheunert and Krzywanski [1926]. The variations in hæmoglobin concentration did not exactly correspond to that of the volume  $pc$  of  $rb$ .

The scatter diagram (fig. 2) indicates a greater increase in  $Hb$  concentration in relation to the increased volume  $pc$  of  $rb$ . The correlation coefficient calculated for the increase of  $Hb\ g\ pc/V_{pcrb}$  ratio in relation to volume  $pc$  of  $rb$  proved the significance of this observation ( $r=0.6875$ ,  $p$ =less than 0.01 [Fisher's Statistical Methods, 1941, p. 202]).

Fig 3 shows that the relative viscosity plotted against volume p c of r b c tends to rise in a greater degree with the increased r b c concentration in horse blood than in ox and sheep blood. That this is not caused by the use of different apparatus is indicated by three points obtained on sheep blood of different r b c volume p c with the present apparatus. They agree well with the data obtained previously with the Ostwald viscometer.

It is therefore probable that the higher viscosity of horse blood (at

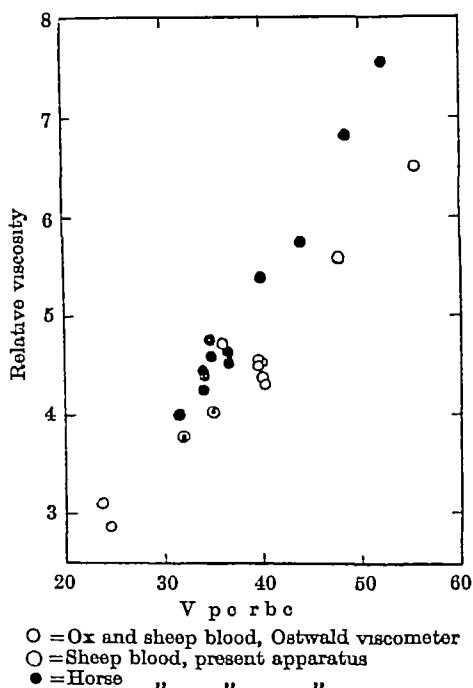


FIG 3—Relations between the r b c concentration and the relative viscosity measured in ox and sheep blood with the Ostwald viscometer (O), and in sheep (O) and horse (●) blood with the viscometer used in the present investigation.

similar volume p c of r b c) is caused by the plasma viscosity, which is also higher in horse blood. The mean value of horse plasma viscosity is 1.89, whereas that of sheep and ox is 1.60.

There is no doubt that the viscosity of horse blood, because of its high sedimentation rate, cannot be measured without a large error with instruments not allowing for a thorough mixing of the blood during the determination. Trevan [1918] modified the Ostwald viscometer so that the blood could be mixed with a wire in an upper container, but he gave no evidence of the efficiency of this method, the blood which he used had a slow sedimentation rate. Fåhræus and Lindqvist [1931], to avoid sedimentation, used a capillary which was rotated during the time

*Viscosity*—The results of determinations of the apparent relative viscosity of plasma and of blood at 38°C carried out with the arrangement shown in fig 1 are given in Table I. Comparison with the estimations

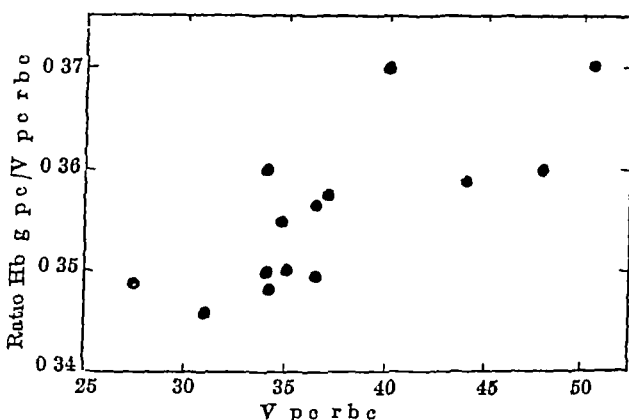


FIG 2—Relation between the r b c concentration and the value of the ratio  $\text{Hb g p c} / \text{V p c r b c}$

made with the Ostwald viscometer on ox and sheep plasma and blood (Table II)<sup>1</sup> shows that the viscosity of the horse plasma and blood is higher than that of the ox and sheep

TABLE II—RELATIVE VISCOSITY OF OX AND SHEEP PLASMA AND BLOOD MEASURED AT 38° C

No	Relative viscosity of plasma	Relative viscosity of blood	V p c r b c	Remarks
1	1.65	4.50	39.8	Ox blood, Ostwald's viscometer
2	1.43	4.30	40.5	" " "
3	1.57	4.56	39.8	" " "
4	1.55	3.10	23.7	" " "
5	1.55	6.54	55.6	" " "
6	1.55	4.37	40.0	" " "
7	1.55	2.87	24.8	" " "
8	1.48	4.52	40.1	" " "
9	1.65	4.24	38.6	" " "
10	1.65	4.72	36.0	" " "
11	1.54	4.52	40.0	Sheep blood, " "
12	1.78	5.56	48.1	" present apparatus
13		4.02	35.0	" " "
14		3.80	31.8	" " "

<sup>1</sup> These data were collected by Miss Banister in the course of Fegler and Banister's work [1946] on conditions influencing the exchange of oxygen in blood *in vitro*



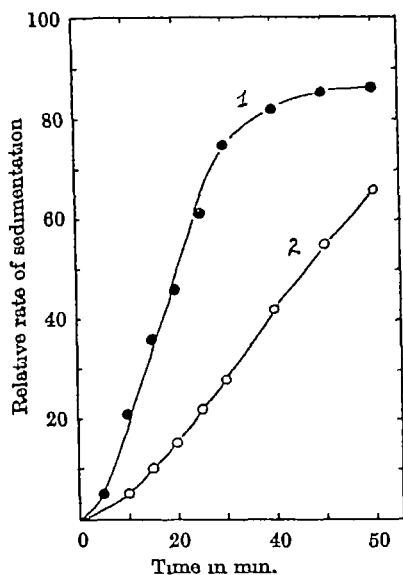


FIG 4—Relative sedimentation rate of heparinised (1) and defibrinated (2) horse blood.

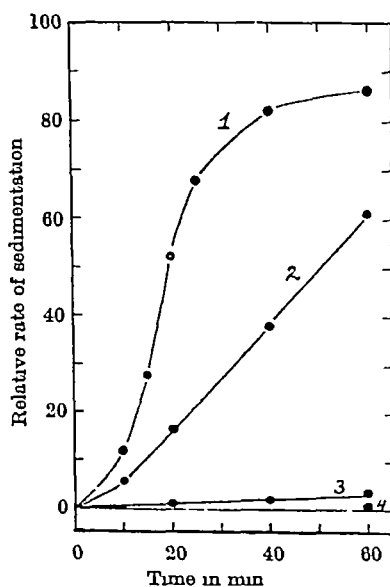


FIG 5—Effect of plasma dilution on relative sedimentation rate 1=100 p.c. plasma, 2=66 p.c. plasma, 3=50 p.c. plasma, 4=r.b.c. suspension in phosphate buffered saline

of determination, but they worked on normal human blood (slow sedimentation rate), and also did not estimate the efficiency of their method

The degree of error which may be caused by the high sedimentation rate of r b c was evident from some determinations made with the present arrangement. If the movement of the container was stopped only for 90 seconds before the actual determination, an increase of about 50 p c in viscosity was obtained in comparison with the determination carried out at constant movement of the apparatus

*Sedimentation Rate of r b c* — In Table I the maximum velocities of sedimentation given are calculated from the continuous curves of relative rate. The curves had a regular S-shape, indicating the initial slower rate of sedimentation, followed by the period of maximum velocity and by the final slow period. The importance of aggregation of the r b c on the rate of sedimentation can be distinctly observed in horse blood. At the beginning the upper surface of the r b c column in the sedimentation tubes is sharply limited, then the limit becomes less and less distinct and the aggregates become visible floating in plasma over the more dense layer. At the same time almost over the whole length of the tube the separation of the r b c column from the walls of the tube becomes apparent. As the cells settle down these phenomena gradually disappear and again, as in the initial stage, the limit between plasma and cells columns becomes sharp, and the granulated appearance of the lower layers also disappears. These phenomena make the determination of the maximum velocity of sedimentation in horse blood only approximate, and this is one reason why the method of making a continuous curve of the sedimentation rate should be preferred for such cases. The continuous curve gives some integration in the period of maximum velocity when also the aggregates formation is the most intense.

It is known that different fractions of the plasma proteins differ in importance in their influence on sedimentation rate. Fåhræus [1929] showed that fibrinogen is the strongest factor. This is illustrated for horse blood by the experiments (fig 4), in which the sedimentation rate was determined on two suspensions of equal r b c volume p c in plasma (1) and in serum (2).

The dilution of plasma with phosphate buffered saline also decreases the sedimentation rate (fig 5).

In connection with these experiments the determinations of the rate of sedimentation of the horse, ox, and sheep r b c in homo- or hetero-geneous plasma were carried out. The results are given in Table III.

They confirm the earlier results of de Haan [1918] and Linzenmeier [1921] showing that the plasma of horse blood is not the only factor causing a fast sedimentation of horse r b c. If it were not so, ox or sheep r b c should acquire the ability to sediment in horse plasma as

quickly as horse r b c , and also horse r b c should sediment in ox or sheep plasma as slowly as do the r b c of these species Ox and sheep r b c , however, sediment very slowly in horse plasma, and the horse cells still have a great sedimentation rate in ox and sheep plasma though decreased in comparison with the rate of their sedimentation in their own plasma

It seems, therefore, that the red cells are also responsible for fast sedimentation The factors of the red cells governing this property could not be removed even by thorough washing of cells in saline It may be, of course, that even ten times washing of cells in saline does not suffice to deprive them completely of their plasma surface covering A similar arrangement may apply to ox and sheep cells which may conserve after washing a layer of their own plasma preventing aggregation when they are suspended in horse plasma It is possible, however, that this layer of "own" plasma is so intimately attached to the surface of the cells that it could not be removed without damage Anyway, it does not make much difference in sedimentation rate in this "cross" experiment if the cells are not washed at all or if they are washed ten times in saline

## DISCUSSION

The sedimentation of horse r b c is the most difficult of the problems investigated in the present work There is no generally accepted explanation of its mechanism All workers seem, however, to agree that the ability of blood to form rouleaux is in direct relation to the rate of sedimentation Rouleaux formation in horse blood is more pronounced than in other species blood in the sense that they are more extensive and more stable This makes horse blood an excellent material for research on this problem It seems, however, that the methods of investigation are still not adequate, since the mere observation of the sedimentation rate is a very crude method giving only the end effect The other more direct and more quantitative method of estimation of the extent of rouleaux formation should be worked out before an approach to the study of the mechanism of the phenomenon should be attempted

There is no direct evidence, apart from some observations of Fåhræus [1929] on mesenteric blood-vessels, that the sedimentation of r b c is a physiological phenomenon occurring in the blood-vessels of the horse But, assuming that the high sedimentation of horse r b c is not a phenomenon which happens only *in vitro*, the possibility arises of an approach to the sedimentation as an important physiological factor of the horse organism It may cause, for instance, an unequal distribution of r b c to different organs, and it is possible that compensation of this unequal distribution may be governed by cardiovascular mechanisms The phenomenon may be partially responsible for the increase in volume

TABLE III — MAXIMUM SEDIMENTATION RATE OF HORSE, OX, AND SHEEP RBC  
IN HOMOGENEOUS AND HETEROGENEOUS PLASMA

	Unwashed rbc		rbc washed 4 times		rbc washed 10 times	
	Max rate of sedimentation, mm/min	Vpc rbc	Max rate of sedimentation, mm/min	Vpc rbc	Max rate of sedimentation, mm/min	Vpc rbc
Horse rbc in horse plasma	32	400	55	275	11	445
" " ox plasma			25	275		
" " sheep plasma	14	350			08	441
Ox rbc in ox plasma			unmeasurable	275		
" " horse plasma			"	275		
Sheep rbc in sheep plasma	unmeasurable	350			unmeasurable	444
" " horse plasma	"	350			"	451

of r b c is higher than that of ox or sheep blood at corresponding volume p c of r b c , the higher viscosity of plasma being one of the causes

5 Maximum sedimentation rate of r b c in heparinised whole blood of the horse varied from 0.6 mm to 5.9 mm per min , depending on the r b c concentration The sedimentation rate of the horse r b c is also high in sheep or ox plasma, whereas the r b c of these species sediment as slowly in horse plasma as in their own

6 The physiological significance of the high sedimentation rate of the horse r b c was discussed

# ACKNOWLEDGMENT

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p c of r b c in the venous blood which was found after exercise. Naturally it is recognised that an important factor is the mobilisation of r b c stored in the spleen [Sheunert and Krzywanek, 1926]. The function of the spleen, however, or of any blood depot, is indicated by the increase in the concentration of r b c from a normal level to one above normal as in exercise. What is worthy of attention in our observations is the relatively low concentration of r b c in a large vein like the jugular in the horse at rest and the increase in their concentration after exercise, but only to the normal level of other animals (about 40–45 V p c r b c). The sedimentation of r b c *in vivo* in the horse blood-vessels is therefore suggested rather by the relatively decreased volume p c of r b c at rest than by the increase in it after exercise, which may be caused not only by increased rate of circulation but perhaps mainly by the contraction of the spleen.

Exercise or excitement may cause in the horse a relatively greater increase in hæmoglobin concentration than in volume p c of r b c (fig. 2). In connection with this it may be mentioned that the author's unpublished experiments on rabbits exposed to low oxygen pressure indicated no change or even decrease in the Hb g p c / r b c V p c ratio. There is therefore a difference in this regard between the reactions of rabbits and horses which may be connected with the difference in the importance of the spleen as a store organ in these two species.

Further experiments on the same lines should be carried out in order to work out in more detail the observations which have been described. The rise in ratio Hb g p c / r b c V p c should be confirmed by more experiments, and the changes in plasma or other factors which may influence the volume of cells should be further explored.

At the present stage it is suggested that increase in the Hb g p c / r b c V p c ratio may indicate the appearance in the circulatory bed of an excited or working horse of the cells containing higher concentration of hæmoglobin. It is highly desirable to discover the origin of these cells.

#### SUMMARY

1 Volume p c of r b c, hæmoglobin concentration, relative viscosity of the plasma and blood, and the sedimentation rate of the r b c have been estimated on 7 healthy horses.

2 Increase in the volume p c of r b c was found in the excited or working horse. The concentration of r b c in blood from the jugular vein in horses at rest is rather low in comparison with other animals.

3 Increase in the Hb g p c / r b c V p c ratio was found in cases with the higher volume p c of r b c with a significant correlation coefficient.

4 Apparent relative viscosity of horse blood at higher volume p c

AN APPLICATION OF BONE-MARROW CULTURES TO  
TOXICOLOGY AND THERAPEUTICS By K HARRISON  
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Station, Porton, near Salisbury

(Received for publication 10th October 1947)

WHEN searching for antidotes to poisons or drugs it is customary to make experiments on the intact animal. In the final stages there is no substitute for animals, because the natural processes of excretion and detoxication tend to work against the antidote, which may reach the site of its action in amounts too small, or too transitory, for demonstrable cure. Provided that the antidote is dramatic in effect, these adverse processes do not weigh heavily, but in border-line cases, and still more in the early stages of an investigation, where a faint clue is all that can be hoped for, it is often desirable to have a different type of approach. Enzyme studies, such as those which led to the discovery of BAL [Peters, Stocken and Thompson, 1945], are the method of choice when specific inactivation is known or suspected. Tissue culture is, in theory, a still better method, because the intact cell can be studied.

Orthodox tissue culture is, however, not free from limitations of a technical kind. Strict asepsis is required throughout, the measurement of growth in three dimensions is very difficult, and the progress of work is slow. In order to avoid these and other handicaps, a simpler method has been worked out, for which the following advantages are claimed: adult tissue from several species of mammal can be examined (orthodox methods rely largely on avian embryonic cells, whose metabolism is peculiar), strict asepsis is not compulsory, results can be obtained within 24 hours, the fatigue of microscopic examination is reduced, and there is a great economy in raw materials. It is not claimed that experiments with intact animals are made superfluous, that a negative result with the method necessarily condemns an antidote, that results with bone marrow apply to all tissues, that *exact* measurements of growth can be made, or that the method is anything but a convenient and rapid test for distinguishing between those remedies which promise well and those which do not.

Although the work described in this paper does not represent an advance in the art of tissue culture as such, it is believed that the method may be of help to toxicologists and those engaged in therapeutic research.





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## METHOD

The femur is taken from an animal as soon after death as possible, successful cultures have been made from marrow taken four hours after death, but for the best results the animal should be killed immediately before the marrow is wanted. Rabbits give the best cultures, but guinea-pigs and rats are also suitable. Experience has shown that the activity of the marrow, as judged by the degree of growth and migration of the granulocytes, is influenced by the time of year, poor growth is obtained in very cold or very hot weather, good growth in the spring and autumn months. The age and sex of the animal make little difference. In order to offset the variations between one animal and another it is advisable, though not necessary, to use an inbred strain of rabbits, and if the animal is bled (10-15 ml, from the ear vein) several days before killing, the marrow cells are usually more active. Bleeding is not necessary during the months when good growth occurs.

With sterile instruments the marrow is carefully removed and put into a sterile petri-dish containing sterile Pannet and Compton Saline (P and C), portions are then transferred to a large microscope cavity slide ( $3\frac{1}{2}'' \times 1\frac{1}{2}''$ , with a cavity  $1\frac{1}{2}''$  in diameter, is a good size) containing P and C, and cut into small pieces not more than 1 mm square, using a sharp cataract knife. The size of the marrow fragments has a considerable influence on the degree of growth, to secure comparable results, it is advisable to cut a large number of fragments and select those of equal size, even so, at least six fragments should be cultured for each part of the experiment. The selected fragments, after washing with P and C to remove debris, are transferred to other cavity slides for exposure to the appropriate reagents, dissolved or suspended in P and C. At no stage must the fragments be allowed to dry.

Afterwards, the fragments are cultured by a simplified "hanging drop" technique. Four sterile test-tubes are placed in a rack, and tilted to an angle of  $45^\circ$  to avoid airborne contamination. A sterile pipette, with teat, is placed in each tube. The first pipette is filled with P and C, the second with serum (taken from the animal, with sterile precautions, just before killing it), and the third with cock plasma, the fourth pipette is for mixing serum and plasma when mounting the fragments. Pieces of sterile glass,  $1\frac{1}{2}''$  square, are set out and covered with petri-dish lids. Culture medium is now prepared by mixing 12 drops of serum and 4 drops of cock plasma. One drop of this mixture is put in the centre of each piece of glass, and three fragments of marrow added (after washing, if necessary, with P and C from the first pipette), in order to hasten clotting and stimulate growth a drop of embryo extract (stored at  $0^\circ$ ) is also added to the medium on the glass, the amount of embryo extract must be carefully controlled, to secure uniform

results (If not readily available, the embryo extract may be omitted, although growth is not then so good.) The drop of medium is now spread to a diameter of about 1 inch with a cataract knife, and allowed to clot, the fragments being arranged with the point of the knife at the corners of an equilateral triangle, with 2-3 mm between each fragment, there is then little danger—even with a very active marrow—that the granulocytes from one fragment will invade the territory of their neighbours. By culturing three fragments on each of two slides, and selecting the mean (by eye), inequalities of manipulation or in the texture of the marrow are smoothed out. Finally, the pieces of glass are inverted over large cavity slides, sealed with vaseline and paraffin wax, and incubated at 37°

With these ordinary precautions there is little trouble from bacterial contamination, as an extra safeguard, the marrow is cut, and the fragments are manipulated, 5 or 6 inches below a slab of plate-glass, which helps to ward off airborne particles. Since the results may be read 16-24 hours after mounting, and since the lag phase of bacterial growth is an appreciable fraction of this time, colonies are rarely seen, and if incubation is carried out for 48 hours or more—which is seldom done, because the wandering granulocytes are by then very much thinned out, and many of them are moribund—the presence of gross contamination does not usually exert a detrimental influence upon the cultures.

The treatment of the fragments in the cavity slides may be varied according to the information sought. As a rule, 6 fragments are preserved in P and C while the other operations go on, they are mounted to serve as controls. Another 6 are put into P and C containing the remedy which is being studied, sometimes, though not always, the behaviour of the "control plus remedy" fragments cannot be distinguished from that of the normal controls, a few trials serve to establish this point, and if the remedy has no effect on normal marrow, in future experiments the "control plus remedy" slides are not prepared. A third set of 6 is placed in a solution of the poison or drug in P and C, the concentration and time of exposure needed to produce a reasonable amount of damage being ascertained by trial and error. Generally speaking, if hydrolysis is not a complicating factor it is better to soak the fragments in a low concentration for a long time than in a high concentration for a short time, because a more regular penetration then takes place, and the retardation of growth and migration is more uniform from fragment to fragment.

The remedy may be studied from the aspects of prophylaxis or cure. In prophylaxis, the remedy is dissolved in P and C, and the fragments soaked in the solution for an appropriate time, quickly washed twice with P and C (using a sterile pipette to drain and fill the cavity slide), and then treated with the poison. After another wash with P and C,

are mounted When attempting a cure, the fragments are exposed to the poison and, after washing, may be dealt with in one or both of two ways they can be soaked in the remedy, washed and mounted, or they can be washed and mounted directly, with a small amount of the remedy in the medium, allowing a longer time for action, or they can be treated by a combination of both methods If a new remedy is being explored, each method should at first be pursued separately

### *BAL and BAL-Intrav as Antidotes to Arsenic*

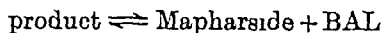
The effect of BAL on damage produced by lewisite oxide is shown in Pl I, A Lewisite oxide is very poisonous, the marrow cells are almost killed by soaking in a M/300,000 solution for 1 hour, but can be revived by mounting in medium containing a nominal concentration of M/10,000 BAL (The concentration of BAL is nominal because this compound is prone to oxidation, and an estimate of available -SH groups could not well be made under the conditions of these experiments) M/10,000 BAL in the medium does not exert any toxic effect Two other observations are worth noting the recovery brought about by BAL appears to be lasting, because the fragments pour out very active and lively granulocytes, and if kept for 48-72 hours begin to develop fibroblasts, and glutathione, a monothiol of natural occurrence, is of no value

BAL-Intrav was developed by Danielli *et al* [1946] with the object of providing a dithiol antidote to arsenicals which should be less toxic than BAL A comparison of toxicity was made between solutions of nominal molarity, freshly made up in medium, since BAL-Intrav appears to penetrate the cells more slowly than BAL, and continued exhibition is necessary when either toxic or therapeutic effects are to be shown clearly BAL-Intrav seems to be about 20 times less toxic than BAL to bone-marrow fragments To make a fair comparison of therapeutic power, BAL and BAL-Intrav were compared in the molar ratio of 1 : 20, because the highest dosages of both substances, when used in animals, approach the toxic level Pl I, B and C, is typical of our findings, the range of effective therapeutic concentrations is smaller with BAL-Intrav than with BAL Even if the molar concentration of BAL-Intrav in the medium was raised to 30 times that of BAL, recovery in all cases was distinctly poorer than with the latter substance If the poisoned fragments, instead of being cultured in medium containing BAL or BAL-Intrav, were soaked in saline solutions of the drugs for 30 minutes, and then cultured in normal medium, BAL produced good recovery whereas BAL-Intrav did not Since BAL-Intrav was designed to penetrate cells less readily than BAL, this result is not to be wondered at, if the rate of diffusion of a healing drug into the cell is reduced,

there must be some sacrifice of therapeutic efficiency, so far as cellular recovery is concerned

Mapharside is a proprietary arsenical used in the treatment of syphilis, BAL and BAL-Intrav are possible antidotes to the signs which are occasionally accidents of treatment. Compared with lewisite oxide, Mapharside is of course far less toxic. Fragments of marrow poisoned with Mapharside (M/2000 for 30 minutes), and cultured in a medium containing M/2000 and M/10,000 BAL, gave a curious and unexpected result, neither concentration produced a satisfactory recovery, and the weaker was more effective than the stronger. In a repetition of this experiment the poisoned fragments were divided into three groups: the first was cultured in normal medium, the second in medium containing M/2000 BAL, and the third soaked in a saline solution of M/2000 BAL for 30 minutes and then cultured in normal medium. Whereas the fragments of the third group showed quite good recovery, those in the second group were less satisfactory although the BAL had a longer time to exert its action (Pl I, D). The inference was made that the reaction product of BAL and Mapharside is as poisonous as Mapharside itself, when the fragments poisoned with Mapharside are soaked in a solution of BAL, the reaction product can diffuse into the solution, and on transferring them to normal medium they are largely freed from the poisonous action of the product, but if the poisoned fragments are mounted in a medium containing BAL, the product remains in the medium and inhibits growth.

It was found that equimolar solutions of Mapharside and BAL, when mixed, did seem to be toxic. Meanwhile, however, Dr L A Stocken had prepared a pure crystalline specimen of the reaction product of BAL and Mapharside. With this material it was possible to show, first, that the product is slightly more toxic than Mapharside (Pl II, A and B), and secondly, that when treated with an excess of BAL the damage caused by the product can be partly reversed (Pl II, C), both results have been independently demonstrated by Peters and Stocken [1947], using rats. It is reasonable to suppose that the product hydrolyses in the cell into its two constituents



If the product is non-poisonous, and becomes poisonous when broken down in the cell, it is also reasonable to suppose that an excess of BAL will tend to shift the equilibrium to the left, Pl II, C, suggests that this process, or something like it, does occur. The importance of using therapeutically high dosages of BAL, when treating signs due to Mapharside, is evident.

Fragments poisoned with Mapharside were not revived by soaking in BAL-Intrav, which is only to be expected, with BAL-Intrav in the medium there was some revival. (The reaction product between BAL-

Intrav and Mapharside was not available as a pure crystal, a mixed solution of BAL-Intrav and Mapharside, in equimolar quantities, seemed to be less toxic than Mapharside alone) When glutathione (M/500) was added to the medium together with BAL-Intrav the revival was better still Danielli *et al* [1946] have suggested that arsenical therapy with BAL-Intrav might be improved by thiols which could act as "carriers" The glutathione effect, however, does not seem to be due to a "carrier" property of the molecule, for a dithiol of proved antidotal value, such as BAL itself, should be more effective than a monothiol in aiding the transfer of arsenical from the protein to the BAL-Intrav, with Mapharside, as we have seen, there is evidence that the BAL-Mapharside product is unstable, and this instability should favour the exchange Yet all attempts to improve the antidotal action of BAL-Intrav by adding BAL have failed It should be noted, however, that in these experiments the dithiols were present together in the medium, from which no excretion was possible, we cannot safely conclude that treatment with BAL would not improve the action of BAL-Intrav in the intact animal, the experiments merely do not support the "carrier" theory Danielli *et al* [1946] have found that in animals a combined dosage of BAL and BAL-Intrav is more efficacious than BAL-Intrav alone, this may well be so, not because of any "carrier" effect, but because with BAL-Intrav the range of effective therapeutic concentrations is small (as noted above), on the other hand, the "carrier" effect may only become important when there is a flow of fluid through a tissue

Glutathione does not act simply as a protective agent for the -SH groups of BAL-Intrav, since neither cysteine, nor ergothioneine, nor BAL itself act in the manner of glutathione when present in the medium with BAL-Intrav Experiments with normal marrow have convinced us that glutathione stimulates the granulocytes themselves

#### *BAL and BAL-Intrav as Antidotes to Mercury and Antimony*

The comparison between BAL and BAL-Intrav has been extended to mercury and antimony Fragments poisoned with mercuric chloride (M/10,000 for 30 minutes) are revived to some extent by BAL-Intrav and to a lesser extent by BAL (Pl II, D) Professor R A Peters suggested that mercury poisoning might be reversed by sodium sulphide, it will be seen (Pl III, A) that sodium sulphide is at least equal to BAL-Intrav if not rather better, a combination of the two does not, however, offer any advantage over either component alone

The illustrations refer only to treatment by soaking, almost identical results have been obtained with the remedies at a lower dilution in the medium The impression has been gained that BAL-Intrav is distinctly superior to BAL in the treatment of mercury poisoning

Later work [1945-46] by W T Longcope and J A Leutscher, reported by Gilman [1946], suggests that BAL-Intrav is a better remedy than BAL for mercury poisoning in animals

Antimony has a wide application in the treatment of tropical diseases due to *Leishmania*, owing to its slighter toxicity, as compared with arsenic, antimony gives rise to fewer complications and sequelæ, nevertheless, a remedy against overdosage is desirable The first experiments were carried out with antimony potassium tartrate, here, as with Mapharside, the reaction product with BAL is toxic, curiously enough, the reaction product with BAL-Intrav is also toxic (Pl III, B and C) Neostam, a proprietary antimonial, gave similar results, the reaction products with both antidotes being toxic, BAL-Intrav was superior to BAL in the soaking experiments (Pl III, D) but slightly inferior in the medium Our findings suggest that provided a free excretion of the reaction products can be secured, by diuretics and a liberal supply of fluid, BAL-Intrav should be of considerable value in antimony intoxications

### *Benzene Poisoning*

Dr Smith Freeman, of Northwestern University, suggested that the culture of bone-marrow fragments might throw light on the mechanism of benzene poisoning Although an extended investigation was not possible, the results are not without interest Benzene itself, suspended as droplets in the medium, is not toxic In the intact animal, where benzene produces (among other symptoms) severe damage to the bone marrow, we may suppose that an oxidation product is the responsible agent, indeed, Yant *et al* [1936] have demonstrated an increased excretion of phenolic substances when benzene is inhaled by animals and man Phenol is toxic, when exhibited in the medium, at a concentration of M/100, the trihydric phenols, phloroglucinol and pyrogallol, are toxic at concentrations of M/100 and M/50 respectively, but the dihydric phenols are very much more toxic, hydroquinone at M/800, catechol at M/400, and resorcinol at M/100 Jost [1932] has isolated dihydric phenols from the urine of animals exposed to benzene vapour, the results with bone-marrow cultures point strongly to the dihydric phenols, rather than phenol itself, being a cause of leucopenia in benzene poisoning

### SUMMARY

1 A method of cultivating bone marrow *in vitro* is described, together with its applications to toxicology and therapeutics

2 BAL and BAL-Intrav have been compared as antidotes to arsenicals BAL-Intrav is less toxic than BAL, but is less potent as an

antidote to lewisite oxide, and the range of effective therapeutic concentrations is smaller. The reaction product of BAL and Mapharside is toxic, the toxicity is diminished by an excess of BAL. The reaction product between BAL-Intrav and Mapharside appears to be less toxic.

3 Extending the comparison to mercury and antimony, it appears that BAL-Intrav is superior as an antidote to BAL. Both substances form toxic reaction products with antimony potassium tartrate and with Neostam, it is suggested that in the intact animal every effort should be made to encourage the excretion of these products.

4 Benzene is not toxic to bone marrow, of its oxidation products, the dihydric phenols are much more toxic than the trihydric phenols or than phenol itself.

We are indebted to Surgeon-Lieut N M Hancox, R N V R, for allowing us to make use of his work on the culture of bone marrow, to Mr P D Mitchell, Dr L N Owen, and Dr L A Stocken for gifts of chemicals, to Mr R M Weston, F R P S, for the photographs, to Professor R A Peters, F R S, Professor G R Cameron, F R S, and Surgeon-Captain A Fairley, R N, for advice and criticism, and to the Chief Scientist, Ministry of Supply, for permission to publish this paper, the substance of which has already appeared in Porton Reports (1944-45).

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## EXPLANATION OF PLATES

(Photographs by dark ground illumination, magnification 5 diameters )

## PLATE I

- A 1 Typical normal control  
2 Poisoned with M/300,000 lewisite oxide for 1 hour, and mounted in normal medium.  
3 As above, but mounted in medium containing M/10,000 BAL
- B 1, 2, 3 As A 2, but mounted in medium containing M/1000, M/4000 and M/16,000 BAL respectively
- C 1, 2, 3 As A 2, but mounted in medium containing M/50, M/200 and M/800 BAL Intrav respectively
- D 1 Poisoned with M/2000 Mapharside for 30 minutes, and mounted in normal medium.  
2 As above, but mounted in medium containing M/2000 BAL  
3 As D 1, but soaked in M/2000 BAL for 30 minutes, and mounted in normal medium

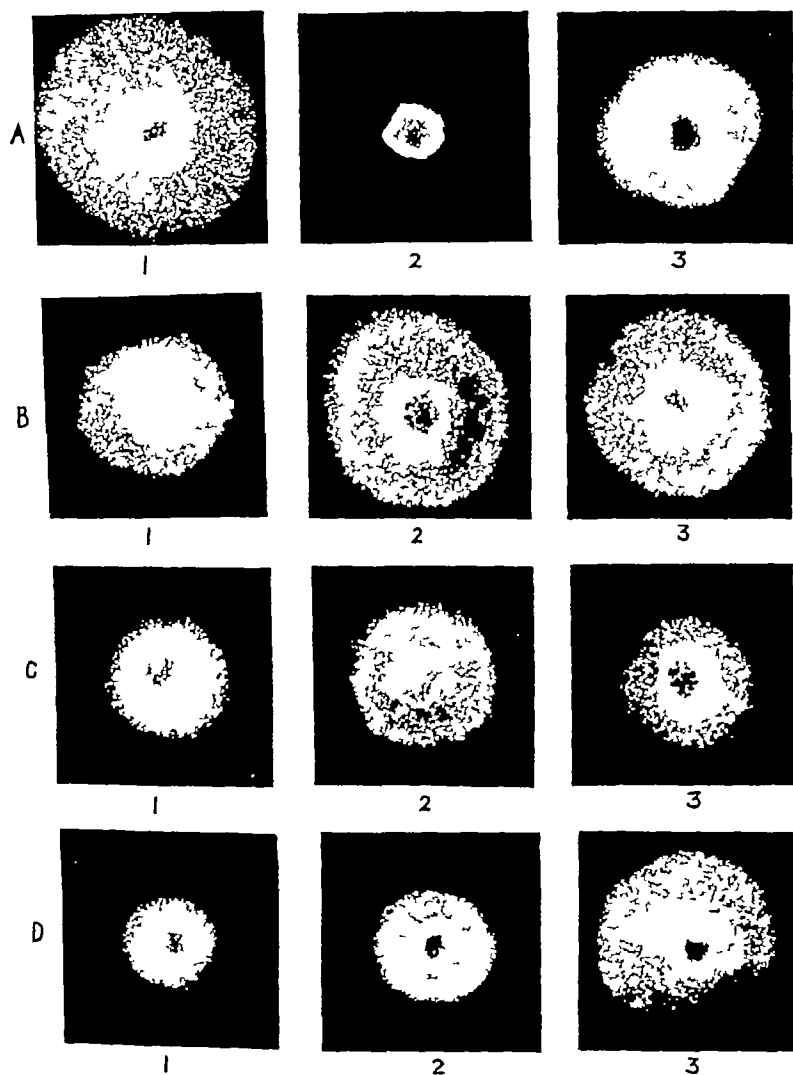
## PLATE II

- A 1, 2, 3 Poisoned with BAL-Mapharside reaction product, M/10,000, M/20,000 and M/40,000 respectively, for 30 minutes, and mounted in normal medium.
- B 1, 2, 3 Poisoned with Mapharside, M/10,000, M/20,000 and M/40,000 respectively, for 30 minutes, and mounted in normal medium
- C 1 Poisoned with M/1000 BAL Mapharside reaction product for 30 minutes, and mounted in normal medium.  
2 As above, but mounted in medium containing M/4000 BAL  
3 As C 1, but soaked in M/2000 BAL for 30 minutes, and mounted in normal medium
- D 1 Poisoned with M/10,000 mercuric chloride for 30 minutes, and mounted in normal medium.  
2 As above, but soaked in M/400 BAL for 30 minutes, and mounted in normal medium  
3 As D 1, but soaked in M/40 BAL Intrav for 30 minutes, and mounted in normal medium.

## PLATE III.

- A 1 As Plate II, D 1, but soaked in M/200 sodium sulphide for 30 minutes, and mounted in normal medium.  
2 As Plate II, D 1, but soaked in a mixture of M/40 BAL-Intrav and M/200 sodium sulphide for 30 minutes, and mounted in normal medium.
- B 1 Poisoned with M/400 antimony potassium tartrate for 30 minutes, and mounted in normal medium  
2 As above, but mounted in medium containing M/4000 BAL.  
3 As B 1, but soaked in M/400 BAL for 30 minutes, and mounted in normal medium
- C 1 As B 1, but mounted in medium containing M/200 BAL-Intrav  
2 As B 1, but soaked in M/40 BAL Intrav for 30 minutes, and mounted in normal medium.
- D 1 Poisoned with M/8 Neostam for 30 minutes, and mounted in normal medium.  
2 As above, but soaked in M/400 BAL for 30 minutes, and mounted in normal medium  
3 As D 1, but soaked in M/40 BAL-Intrav for 30 minutes, and mounted in normal medium







NOTE ON A METHOD FOR THE DEMONSTRATION OF  
PULMONARY VASOMOTOR FIBRES By I DE BURGH  
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University of Edinburgh

*(Received for publication 17th December 1947)*

WHEN the blood inflow to the lungs is kept constant and there is a free outlet for the blood from the left auricle, the response of the pulmonary arterial pressure to nerve stimulation is an effective measure of pulmonary vasomotor activity provided broncho-constriction is not an accompanying response. The passive effects on the pulmonary vascular bed due to broncho-constriction can be prevented by carrying out nerve stimulation whilst the lungs are quiescent, or by the addition of atropine to the blood if pulmonary sympathetic vasomotor responses alone are being tested. These conditions can be achieved by perfusion of the pulmonary vascular bed with additional perfusion of the bronchial arteries or of the whole systemic circulation [Daly, Elsdon, Hebb, Ludány and Petrovskaja, 1942]. Such perfusion experiments, although giving the desired control conditions, are somewhat laborious. A more simple procedure for testing the presence of adrenergic but not cholinergic pulmonary vasomotor fibres has been developed whereby control of the pulmonary circulation is established by perfusion through one lung only of an atropinised animal.

#### METHODS

In dogs anaesthetised with intravenous chloralose (British Drug Houses, Ltd), 0.1–0.15 g/kg body-weight, a tracheal cannula is inserted and artificial respiration carried out by a Starling Ideal pump. The sternum is split in the mid-line and the thoracic viscera exposed. The left pulmonary artery is carefully freed by blunt dissection from adjacent tissues and two loose ligatures placed round the artery in its intrapericardial course. In order to obtain a sufficient length of artery the ductus arteriosus may have to be divided. Care is taken to exclude nerve fibres from within the ligatures to ensure that the pulmonary vasomotor path to the left lung is not interrupted. Heparin (B D H, Jorpes), 1000 International Units per kg body-weight, is injected intravenously. A cannula is inserted into the right auricle through the

appendix and connected by rubber tubing to the input side of a Dale Schuster pump chamber (fig 1) The ligature on the left pulmonary artery nearest to the main branch is then tied, and gentle traction exerted upon the remaining loose ligature to prevent air entering the vessel whilst the portion of the artery between the two ligatures is incised and a cannula inserted and tied in This cannula is connected by rubber tubing to the output side of the pump chamber The pump chamber and connections having been filled with blood from the right

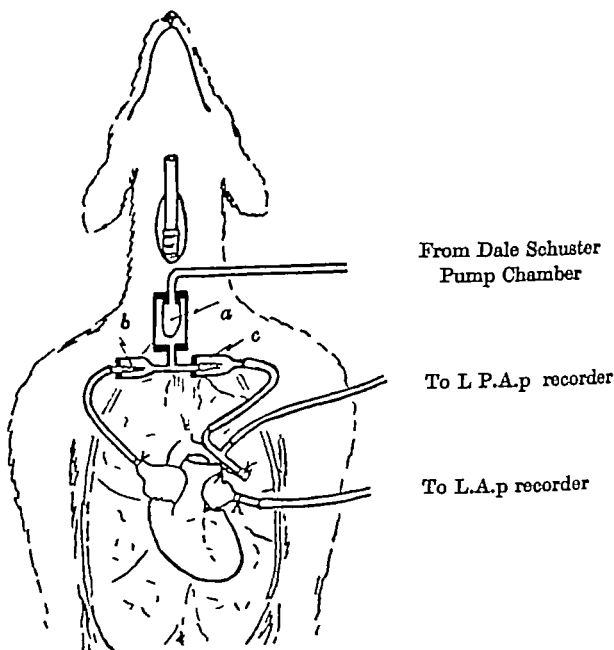


FIG 1—Arrangement of perfusion apparatus  
*a*=rubber finger stall in pump chamber  
*b* and *c*=inlet and outlet valve chambers respectively

auricle or from another animal bled for the purpose, the pump is started up and the left lung perfused We performed two experiments, one in which the procedure was carried out as described above, and the other in which a small water-jacketed blood reservoir kept at 37° C was inserted between the right auricle cannula and the input side of the pump chamber

The following records were taken left carotid arterial pressure, left auricle or right auricle pressure, left pulmonary arterial perfusion pressure, and respiratory efforts of the animal by a lever attached through a thread to the ribs

Electrical stimulation was carried out by a square-wave stimulator

with independent control of voltage duration and frequency of current [Ritchie, 1944] In the following description of changes in these parameters, the numerical values of the voltage, duration and frequency will be placed in that order, separated by an oblique stroke thus, a stimulus of 10 volts, 1 m/sec duration and 50 per sec frequency = 10/1/50

For the demonstration of pulmonary vasomotor nerve activity the method has limitations which it is important to recognise Since the left pulmonary artery blood-flow is kept constant, any immediate changes in left pulmonary arterial pressure following nerve stimulation can only be due (1) to alterations in the resistance of the pulmonary vascular bed of the left lung, or (2) to changes in left auricle pressure, or (3) to variations in the amount of blood transferred through communicating vessels from the bronchial vascular system to the pulmonary vascular bed of the left lung The first of these can be due to direct electrical excitation of the pulmonary nerve fibres, or to excitation of their cells of origin such as might occur by an ischaemic or reflex stimulus due to a fall in systemic arterial blood-pressure consequent upon concomitant excitation of cardio-inhibitory fibres The cervical and thoracic vagosympathetic nerves contain both cardio-inhibitory and pulmonary vasomotor fibres Atropinisation of the animal prevents the cardio-inhibition and blood-pressure fall. The second can be due to excitation of cardio-accelerator or cardio-inhibitory fibres, which generally leads to a fall and rise of left auricle pressure respectively If the direction of pressure changes in the left auricle and left pulmonary artery is similar, it is clear that the pulmonary arterial pressure alteration may be entirely dependent upon the change in pressure in the left auricle If, however, these pressures move in opposite directions, the direction of the left pulmonary arterial pressure change is not determined by that in the left auricle Here again atropine will prevent such unwanted cardiomotor events The third effect which may alter the pulmonary arterial pressure is a change in the amount of blood transferred from the bronchial to the pulmonary vascular bed This may occur as a result of gross alterations in systemic arterial pressure [Berry and Daly, 1931] There is also the possibility that even in the absence of blood-pressure changes, pulmonary nerve stimulation may control the calibre of the communicating vessels between the two systems [Daly and Hebb, 1942]

## RESULTS

The foregoing considerations underline the necessity for the administration of atropine when using the method for testing pulmonary vasomotor nerve fibres in nerve trunks which also contain cardio-

inhibitory and broncho-constrictor fibres. This may be illustrated by reference to fig 2. Before the administration of atropine, stimulation of the left cervical vagosympathetic nerve (LCVS) at 40/1/50 at

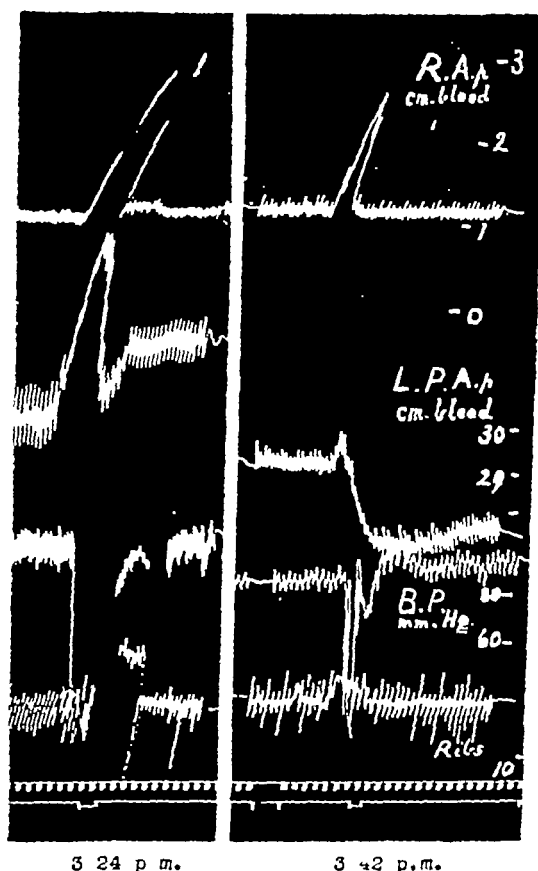


FIG 2—Expt 85a Dog, ♂ 10.2 kg Chloralose Heparin Left lung perfused Right lung normal circulation. LCVS (left cervical vagosympathetic nerve) cut Left lung perfusion started 2.49 p.m. Rectal temp 37°–37.5° C

3 24 p.m Stimulation of LCVS 40/1/50

3 42 p.m Stimulation of LCVS 30/1/50

Tracings from above downwards

R.A.p = right auricle pressure

L.P.A.p = left pulmonary arterial pressure

B.P. = carotid arterial pressure

Ribs = rib movements

3 24 p.m. causes a rise in left pulmonary arterial perfusion pressure (L.P.A.p), which may be due in part to an increase in left auricle pressure (L.A.p) consequent upon the heart slowing. The record, in point of fact, shows a right auricle pressure (R.A.p) rise, but since the heart slowed it can be safely assumed that the L.A.p also rose. An



additional explanation of the rise in L P A p is the production of an ischaemic or reflex stimulation of the pulmonary vasoconstrictor nerve-cells of origin. Later on in the experiment at 3.42 p.m. a weaker stimulus 20/1/50 is followed by a fall in L P A p. The R A p rise is smaller than before, and if we assume that the L A p is correspondingly changed, then the production of the L P A p fall cannot be due to

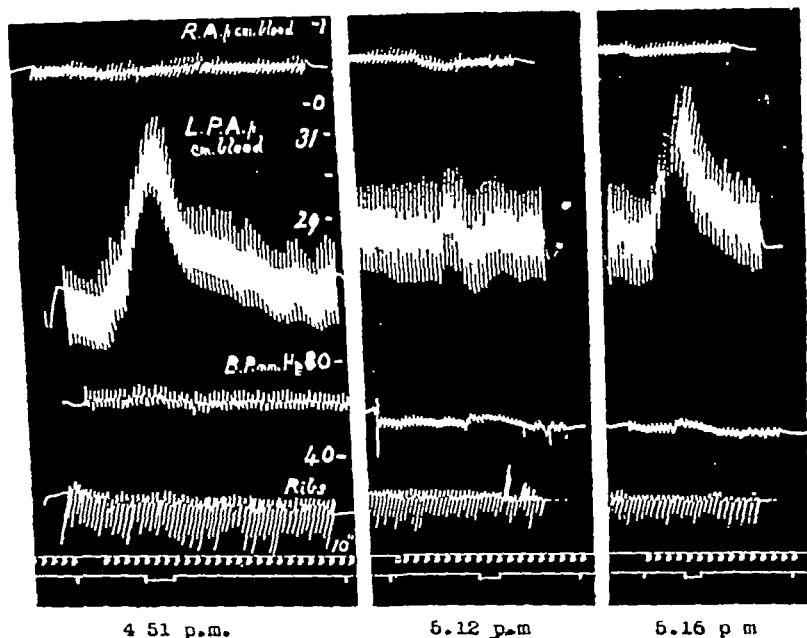


FIG 3—Same experiment as fig 2

4.10–4.30 p.m. Atropine sulphate 7 mg  
 4.51 p.m. Stimulation of L T V S 40/1/50  
 5.12 p.m. Stimulation of R T V S 50/1/50  
 5.16 p.m. Stimulation of L T V S 40/1/50  
 T V S = thoracic vagosympathetic nerve

events taking place in the left auricle. It may have been due in part, however, to the transfer by the communicating vessels of a smaller amount of blood from the bronchial to the pulmonary vascular bed on account of the rapid and profound drop in systemic blood-pressure which is recorded. Thus it may be seen how difficult it is to determine the precise origin of the changes in L P A p which have been produced in the two tests and, although we may suspect that stimulation of vasoconstrictor nerve fibres causes part of the one response (L P A p rise) and stimulation of vasodilator fibres part of the other (L P A p fall), we have no decisive evidence that this is so. The administration of atropine, on the other hand, provides conditions which are simple to analyse, and enables us to observe pulmonary vascular responses to

nerve stimulation in the absence of any bronchomotor or systemic vasomotor changes which could simulate or mask such responses. For example, in the experiment of fig 3 when atropine sulphate (7 mg) is administered intravenously and a nerve, in this case the thoracic vago sympathetic (T V S) nerve, containing pulmonary vasomotor fibres is subsequently stimulated, the pressure in the right auricle (and presumably in the left auricle) and the systemic arterial pressure show no significant change. In spite of this, stimulation of the caudal end of the L T V S nerve (40/1/50 at 4.51 and 5.16 p m) at the aortic arch level gives a well-marked L P A p rise, and of the caudal end of the R T V S nerve (50/1/50 at 5.12 p m) at the upper border of the superior azygos vein a scarcely significant response, suggesting an ipsilateral distribution of the nerve fibres. Since an intravenous dose of 2 mg atropine is usually sufficient to paralyse broncho-constrictor fibres, passive effects from broncho-constriction on the pulmonary vascular bed can be ruled out.

We interpret this result as indicative of the presence in the T V S nerves of vasoconstrictor nerve fibres to the pulmonary vascular bed or, an alternative which must not be neglected and is dealt with below, of the presence of vasodilator fibres to the blood-vessels in the lung which control the transfer of blood from the bronchial to the pulmonary vascular bed.

Our next step was to obtain a direct measurement of the pressure in the left auricle during pulmonary nerve stimulation. Fig 4 shows that a weak stimulus 5/10/50 of the caudal end of the L T V S nerve at 4.38 p m, eight minutes after the intravenous injection of 2 mg atropine sulphate, gives a small diphasic response of the L P A p without causing any significant change in L A p or in systemic blood-pressure. A stronger stimulus 20/10/50 at 4.48 p m caused a marked rise in L P A p accompanied by a fall in L A p. The lowering of the L A p may have been due to an increase in the resistance of the pulmonary vascular bed, and/or to the cardiac acceleration of 28 to 41 beats in 10 seconds which occurred. If due solely to the cardiac acceleration, the L P A p would tend to fall, and therefore it is the more significant, as indicating that pulmonary vasoconstriction occurs, that the fall in L A p is accompanied by a rise in the L P A p of 18 per cent.

With regard to the possibility mentioned above that the L P A p rise is due to vasodilatation of the communicating vessels between the bronchial and pulmonary vascular bed, nerves accompanying the bronchial arteries have been described by several investigators [Reisseisen and Sommering, 1808, Reisseisen, 1822, Kolliker, 1852, Berkly, 1893, Braeucker, 1926]. Larsell and Dow [1933] demonstrated the presence of nerve fibrils in the smooth muscle of the bronchial arteries, which probably take origin from the thoracic sympathetic

chain at levels T<sup>1</sup> to T<sup>5</sup> [Hovelacque, 1927, Rjasansky, 1928] Concerning the function of these nerves, Daly and Euler [1932] have shown that stimulation of the thoracic vagosympathetic nerves in isolated lung preparations, perfused both through the bronchial arteries and

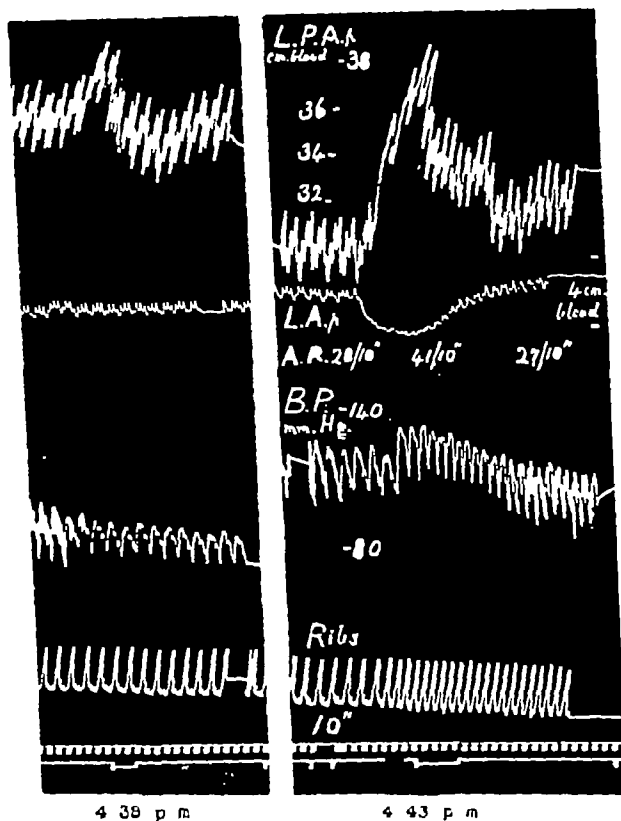


FIG 4—Expt 85b Dog ♀, 11.7 kg Ether induction followed by chloralose  
Left lung perfused Right lung normal circulation Perfusion started 2 12 p.m

4 38 p.m Stimulation of L T V S 5/10/50  
4.48 p.m Stimulation of L T V S 20/10/50  
A.R. = rate of auricle

pulmonary artery, causes a rise in bronchial arterial pressure which is not suppressed by atropine but is suppressed by ergotamine. In other words, there is evidence that such nerve fibres are vasoconstrictor and not vasodilator\*. This is supported by the observation in fig 4 that T V S stimulation at 4 48 p.m causes a small rise in blood-

\* Recently H. D. Bruner and Carl F. Schmidt (*Amer J Physiol*, 1947, 148, 648) have presented data which indicate that the vagus carries cholinergic dilator fibres and the thoracic lumbar predominantly adrenergic constrictor fibres to the bronchial artery system.

pressure which in all probability is due to stimulation of sympathetic vasoconstrictor fibres to the vessels of trachea, cesophagus and thoracic parietes, as well as to the bronchial arteries [Daly and Euler, 1932] If this is the correct interpretation, then we may assume that any effect of T V S nerve stimulation on the bronchial arteries would tend to cause a diminution in blood transferred to the pulmonary circulation, and therefore a fall of L P A p, not a rise. The rise in L P A p which is observed to occur with stimulation of the T V S in atropinised preparations is therefore unlikely to have been due to dilatation of the communicating vessels. We may conclude, then, that it is due to pulmonary vasoconstriction.

We have been able to resolve unequivocally the problem by demonstrating in the perfused living animal that when the systemic circulation pump is temporarily switched off and the blood-pressure thereby reduced to zero, T V S stimulation still causes pulmonary vasoconstriction in the absence of bronchomotor effects. These experiments will be published shortly.

#### SUMMARY

In the atropinised dog in which the left lung only is perfused at constant blood inflow, experiments are described which give evidence of the presence of pulmonary vasoconstrictor fibres in the thoracic ~~vagosympathetic~~ nerves.

AIP

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PULMONARY VASOCONSTRICTION IN RESPONSE TO IN-  
HALATION OF CO<sub>2</sub> IN THE ISOLATED PERFUSED  
LUNGS OF *MACACUS RHEBUS*. By CATHERINE O  
HEBB and R H NIMMO-SMITH From the Department of  
Physiology, Edinburgh University

(Received for publication 6th January 1948)

THE purpose of this communication is to describe briefly the results of some tests which were carried out on the isolated perfused lungs of *Macacus rhesus* which, though limited in number, provide evidence of a marked pulmonary vasoconstrictor action of CO<sub>2</sub>. These results may be of interest in the light of the conclusion reached by Euler and Liljestrand [1946] that CO<sub>2</sub> has a similar action on the pulmonary vessels of the cat. So far as we are aware, however, no study of this kind has been made on the monkey, and our evidence suggests that the pulmonary vessels of this animal are as sensitive, if not more so, to inhaled CO<sub>2</sub> as are those of the cat (as judged by the published observations of the authors cited).

### METHODS

The lungs were perfused with blood at constant volume inflow and under negative pressure ventilation. The procedure and techniques employed are similar in principle to those described by Daly [1938]. The animals used were bled from the femoral artery after administration of nembutal (20 mg/kg injected intraperitoneally) and a local application of novocaine. Heparin was used to prevent coagulation of the blood.

The pulmonary arterial pressure (P A p) was measured by means of a small membrane manometer. The tidal air volume (T A) was recorded by means of a small spirometer equipped with a writing-point. This was connected directly to the bronchial or tracheal cannula, the lungs, spirometer and intervening connections (of wide-bore rubber tubing) formed a closed system of approximately 300 c.c. in capacity. Additions of gases to this system were made by means of a 20-c.c. syringe attached to a fine hypodermic needle the point of which was passed into the airway through the rubber connection nearest the lungs. At each observation the injection of gas (either O<sub>2</sub> or CO<sub>2</sub>) was completed.

during the momentary pause in the respiratory movements which occurred just before the beginning of inspiration. By timing the injection in this way it was possible to ensure that the greater part of the added gas would be carried into the lung with the succeeding inspiration. It is not possible to be certain in what concentration the added gas reached the lungs, if evenly diluted by the volume of air with which it

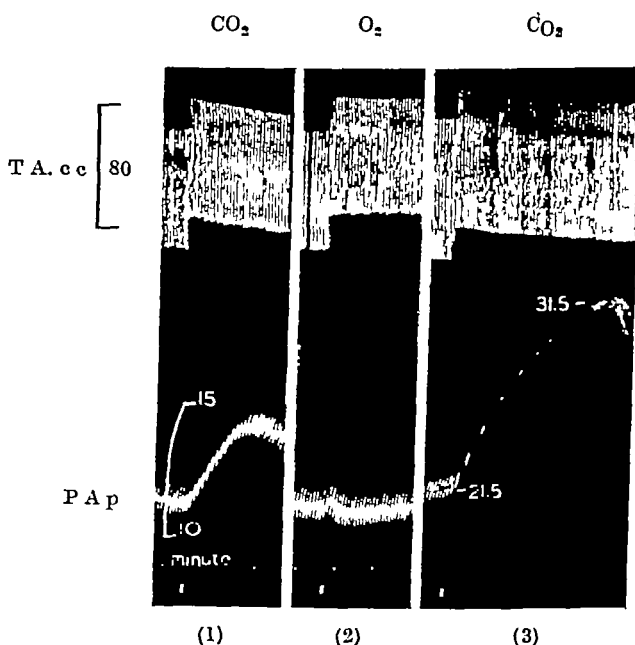


FIG 1—*Macacus rhesus*, 4.1 kg, ♀, 10 yrs old. Isolated right lung perfused with heparinised blood of same animal (open circulation). Observations (1) and (3) at 1 hr 11 min and 3 hr 19 min. after commencing perfusion, 20 c.c.  $\text{CO}_2$  added to closed ventilating system. Observation (2) at 1 hr 27 min., 20 c.c.  $\text{O}_2$  added to system. Each addition of gas indicated by upward displacement of spirometer (top tracing) as well as by signal.

P.A.p. = pulmonary arterial pressure (calibrated in cm blood), T.A. = tidal air

was first carried into the lungs, the concentration of  $\text{CO}_2$  to which the tissue was exposed may have been of the order of 20 to 30 per cent during the space of one or two respirations, i.e. until more adequate mixing of the gas occurred.

## RESULTS

The two figures shown illustrate the results of two out of a total of three experiments. In all three it was found that the addition of  $\text{CO}_2$  to the airway in 20 c.c. doses caused a sharp increase in P.A.p. In the experiment of fig. 1 it may be seen that the injection of 20 c.c. of  $\text{CO}_2$  into

the airway at 1 hr 11 min after the start of perfusion was followed almost immediately (within 5 seconds) by a rapid rise in P A p, which at its maximum was equal to an increase of about 20 per cent above the control value. A similar injection at 3 hr 19 min produced a much larger change, in this case the increase being equal to 47 per cent above the control value. The maximum was reached in both tests between 1 and 2 min after the administration of CO<sub>2</sub>, the rise in pressure was not sustained and returned gradually to the control level.

In the experiment of fig 2 there was evidence again of an increasing

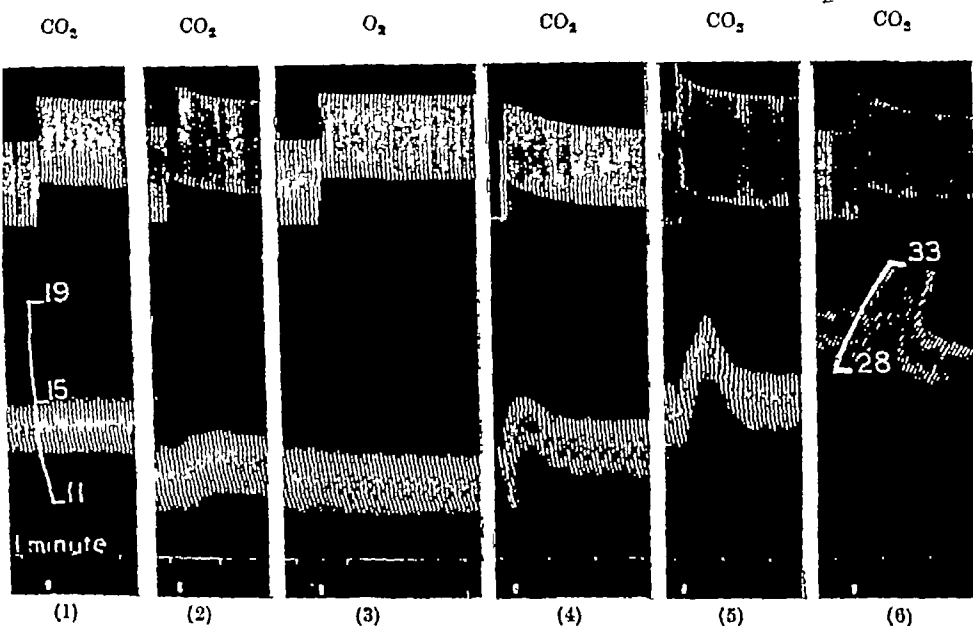


FIG 2—*Macacus rhesus*, 4.1 kg, ♀ 3 yrs old. Both lungs perfused together with closed circuit containing 1 part heparinised blood of animal + 1 part human citrated plasma. Six successive observations made at 1 hr 12 min, 2 hr 12 min., 2 hr 43 min., 3 hr 25 min., 5 hr 1 min., and 5 hr 48 min. from commencement of perfusion. Administration of gases as in fig 1, volume in each case being 20 c.c.

intensity of the response as perfusion proceeded for as long as 3 hours. At the first test at 1 hr 12 min there was a barely perceptible increase in P A p, amounting to about 3 per cent, at 2 hr 12 min an increase of about 9 per cent was obtained, while at 3 hr 25 min an increase of 27 per cent was observed. In this experiment this was the largest order of response observed, since the last two responses after 5 hours of perfusion were smaller, being equal to increases of 18 and 10 per cent (in that order) above the values immediately preceding each addition of CO<sub>2</sub>.

It will be observed that in each experiment the addition of 20 c.c. pure oxygen had either no effect on the P A p (fig 2) or produced a

slight fall (fig 1) These injections served as a useful means of controlling any mechanical effects—such as an increase in intrapulmonary pressure—which might be suspected to play a part in producing the rise in P A p observed when  $\text{CO}_2$  was injected into the airways in a similar manner

An increase in intrapulmonary pressure leading to a rise in P A p may occur as the result of bronchoconstriction [Daly and Hebb, 1942], and in assessing any change in the flow pressure it is necessary to take this factor into consideration In the present experiments we found no evidence that  $\text{CO}_2$  caused a change in the resistance of the airways, since the tidal air volume remained nearly constant over the period of each observation when maximum changes in P A p were produced

We concluded, therefore, that  $\text{CO}_2$  acted specifically to produce pulmonary vasoconstriction While it seemed likely that changes in P A p of the order observed were due to arterial or arteriolar constriction, constriction of the venules or capillaries might also have been implicated [see Daly, 1938] We obtained no evidence by which we could exclude or implicate any particular group of pulmonary vessels in the response

## DISCUSSION

As we have already mentioned, Euler and Liljestrand [1946] found evidence that  $\text{CO}_2$  has a constrictor action on the pulmonary vessels of the cat Their experiments were carried out on living anaesthetised animals Under these conditions it was found that inhalation of gas mixtures containing 6.5 per cent or more of  $\text{CO}_2$  increased the P A p The order of change which they found was not so large as some of the responses observed in the two experiments described here, nor is it definitely proven in their experiments that the effect depended entirely on an increased resistance of the pulmonary vessels, since the coincident rise in systemic pressure was much larger and it would be difficult to exclude the possibility that the increase in P A p was not in some degree dependent upon cardiac or systemic effects It would be interesting, therefore, to compare the blood-vessel responses of the monkey lungs and cat lungs under the same conditions of experiment, since the foregoing considerations suggest that of the two the former may be the more reactive to  $\text{CO}_2$

We should add that we have had the opportunity of making a number of similar tests on the isolated perfused dog lungs (8 experiments) but, although observing the same conditions of test, we were unable to produce any significant change in the P A p We are inclined to believe that this is a true species difference, but it is possible that it may in part have been due to differences in ventilation The perfused monkey lungs showed little or no evidence of spontaneous broncho-



constriction, but in the perfused dog lungs this condition was almost always present to some degree, and may have been sufficient to hinder rapid access of CO<sub>2</sub> to the alveoli. If so, we may suspect that the alveolar concentration of the gas did not reach a very high concentration at any one time, while there is the additional possibility that the vessels were able to adapt themselves to an increase in concentration of CO<sub>2</sub> which was only slowly built up.

#### SUMMARY

Experiments carried out on the isolated perfused lungs show that the inhalation of CO<sub>2</sub> in relatively large amounts leads to a rapid rise in pulmonary arterial pressure which is found to be due to constriction of the pulmonary vessels. This effect was not observed when similar tests were made on the isolated perfused lungs of dogs.

#### ACKNOWLEDGMENT

Part of the expenses of this research were defrayed by a grant from the Moray Endowment Committee, to whom we wish to express our thanks.

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THE EFFECTS OF SKIN CONTAMINATION WITH LIQUID  
MUSTARD-GAS ON WATER BALANCE IN ANIMALS By  
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Station, Porton, Wilts

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WHEN liquid mustard-gas, 2,2'-dichlorodiethyl sulphide, is applied to the skin, local erythema, vesication, and cedema are observed in man, and erythema and cedema in animals. If the dose is sufficiently large histological changes may be evident in lymph glands, lymphoid tissue, and the bone marrow. These changes may lead to leucopænia [Stewart, 1918, Cameron, unpublished results]. Similar observations in the blood-forming tissues have been made after administration of nitrogen mustards [cf Gillman and Phillips, 1946, Cameron, Courtice, and Jones, 1947]. Mustard-gas may also affect the alimentary tract, causing diarrhoea and salivation in experimental animals [Warthin and Weller, 1919]. These observations suggest that mustard-gas or a derivative penetrates the skin and is absorbed into the circulation.

Banks, Bourns, Francis, Hopwood, and Wormald [1946] have shown that mustard-gas containing  $S^{35}$  as a tracer element combines *in vitro* with proteins. It seems possible, therefore, that some of the mustard-gas will combine with the proteins of the tissue fluid after penetration of the skin. If this is so, a portion should enter the lymphatics and be absorbed in this way. The first part of the present investigation concerns the local effects of mustard-gas on capillary permeability and possible absorption by the lymph. The second part deals with the general systemic effects of fluid loss locally in the skin and from the alimentary tract.

The experiments reported here were carried out in 1941-43, in view of the possibility of heavy contamination with liquid mustard-gas during the war, especially in the tropics. The hazard in hot climates is increased by the more rapid penetration of mustard-gas through the hot sweaty skin [cf Cullumbine, 1947].

#### METHODS

Dogs, goats, rabbits, and guinea-pigs have been used in this investigation. All animals were unanæsthetized except where specially mentioned.

The local effects were investigated on dogs anaesthetized with nembutal. The flow and composition of the lymph from the skin of the forepaws were studied by cannulating the main lymph duct just above the paw and collecting the lymph before and after contaminating the paw with liquid mustard-gas. The toxicity of the lymph and of the blood coming from the contaminated area was tested on tissue cultures of bone-marrow fragments [cf Harrison and Randall, 1947].

The general effects of the local loss of fluid on the blood were studied by determinations of the blood volume (Evans Blue method), plasma protein concentration (microkjeldhal), hæmoglobin per cent (Haldane

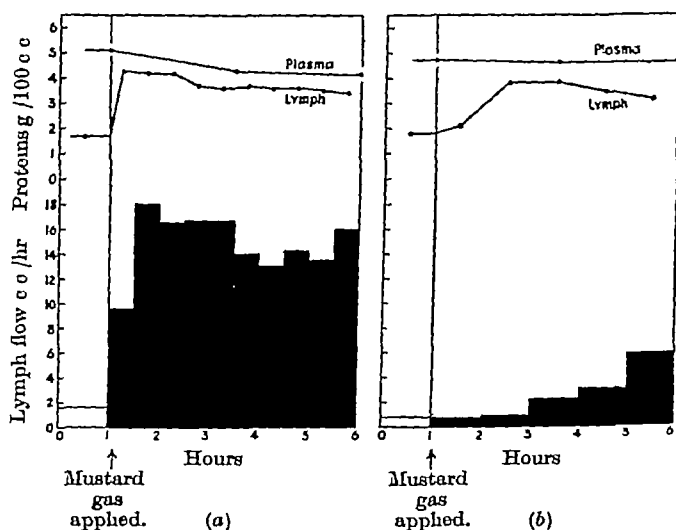


FIG 1—Effects of contamination of the forepaw of the dog with liquid mustard gas, 40 mg/kg body weight, on the lymph from the main lymph duct cannulated just above the paw

hæmoglobinometer), and red cell counts in goats and rabbits. The dehydration caused by the resulting diarrhoea was investigated by determinations of the body-weight and water content of the tissues in rabbits and guinea-pigs. The effects of dehydration on renal function were studied in rabbits and in acute experiments in dogs anaesthetized with sodium barbitone.

## RESULTS

### 1 Local Effects

*The Lymph Flow from the Dog's Paw before and after contamination with Liquid Mustard-gas*—Typical results of these experiments are shown in fig 1. In all cases local oedema was produced with a considerable increase in lymph flow and in lymph protein concentration to

about the level in the plasma. In the experiment represented in fig 1 (a) the onset of oedema was rapid and the lymph flow became spontaneous, and greatly increased soon after the application of mustard-gas, whereas in the experiment represented in fig 1 (b) the onset of oedema and increase in lymph flow was much more gradual

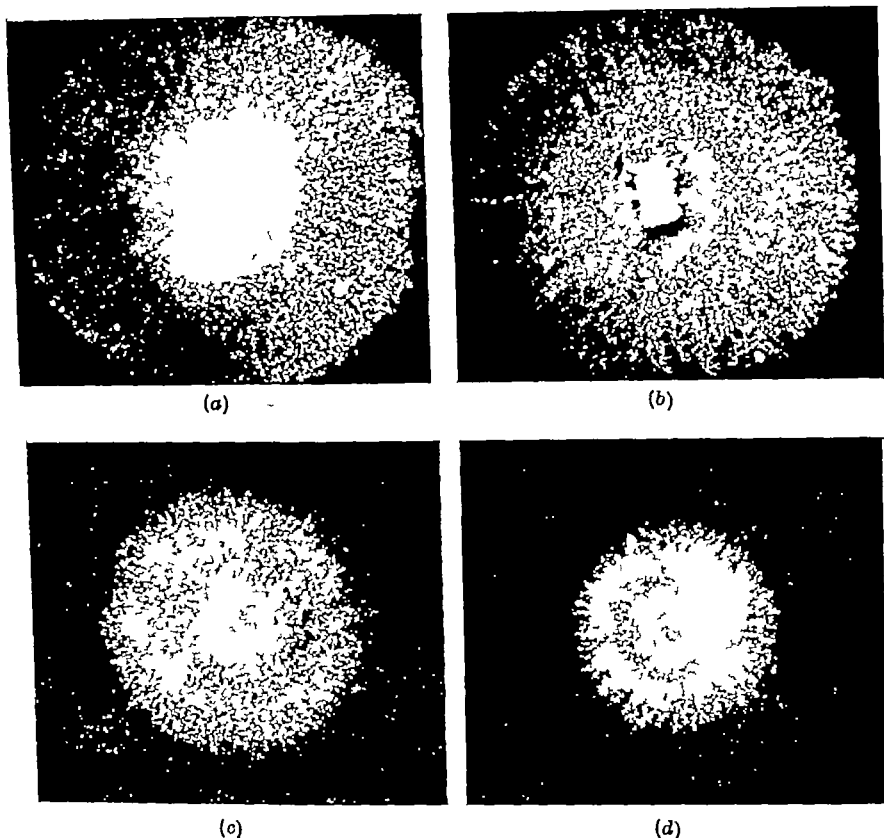


FIG 2—The growth of bone marrow fragments in tissue culture after 24 hours

- (a) Normal bone marrow control.
- (b) Bone marrow previously placed for 1 hour in normal lymph from the forepaw duct of dog
- (c) Bone marrow previously placed for 1 hour in lymph from forepaw duct after contamination of paw with mustard gas
- (d) Same as (c) except that lymph was changed every 10 minutes

Thus although the room temperature at which the experiment was done was always about the same, the rate of penetration of mustard-gas into the skin varied in different dogs, but in all cases permeability of the local capillaries to the plasma proteins was increased

*The Toxicity of the Lymph draining the contaminated Area*—Lymph was collected from the ducts of the paws of dogs before and after the

skin was contaminated with mustard-gas. The toxicity of this lymph and of the blood from the vein draining the paw was tested on the growth of fragments of bone-marrow (rabbit) in tissue culture. The paw lymph and blood taken before contamination we have termed normal lymph and normal blood. In all, five experiments were performed in which 20–40 mg/kg body-weight of mustard-gas was applied to the paw in each case.

Fragments of bone-marrow were placed in normal lymph and normal blood (both whole blood and serum) for 1 hour. Further fragments were placed in blood and serum taken from the vein of the foreleg after contamination and in lymph collected during the first, second, and third hours after application of the mustard-gas. In three experiments the lymph was changed every 10 minutes, so that in each of these tests the fragments were placed for a total of 1 hour in six different lots of lymph. In each individual test five bone-marrow fragments were used. They were all approximately the same size, and in any one experiment were taken from the bone-marrow of the femur of one rabbit. After 1 hour, the lymph, blood, or serum was removed and the fragments cultured for 24 to 48 hours. The growth increment of the fragments was observed at these times.

In all five experiments, the growth of those fragments that had been placed in normal lymph or in blood and serum taken before and after contamination showed no significant difference from that of control fragments cultured without previously being placed in lymph or blood. In four out of the five experiments, the growth of those fragments placed in lymph collected after mustard-gas contamination was considerably decreased, in one case being almost completely inhibited. Typical results are shown in fig 2. These experiments, therefore, indicate that a substance which inhibits the growth of bone-marrow in tissue culture is absorbed in the lymph.

## 2 General Effects

*Local Fluid Loss and Hæmoconcentration*—Typical blood changes caused by fairly large doses of mustard-gas applied to the clipped skin of goats and rabbits are shown in fig 3. Fig 3 (a) shows the average results of skin application of 40 mg/kg on three goats. The loss of fluid locally causes a fall in plasma volume with a resultant hæmoconcentration and decreased plasma protein level. Fig 3 (b) represents average figures for 6 rabbits contaminated with 15 mg/kg. In the rabbit, the degree of hæmoconcentration is less than in the goat, but the fall in plasma protein level is usually considerable, indicating a more effective reabsorption of fluid from the tissues in general. The effects of skin application of mustard-gas on the blood in the dog are seen in fig 7.

As a result of the fall in both plasma volume and plasma protein concentration, the total circulatory plasma proteins fall considerably (Table I). Both the total albumin and globulin fall during the first 24 hours after the application of mustard-gas, and generally return to normal in 3 or 4 days if death does not ensue before this.

The blood pressure in rabbits, measured by the Grant-Rothschild

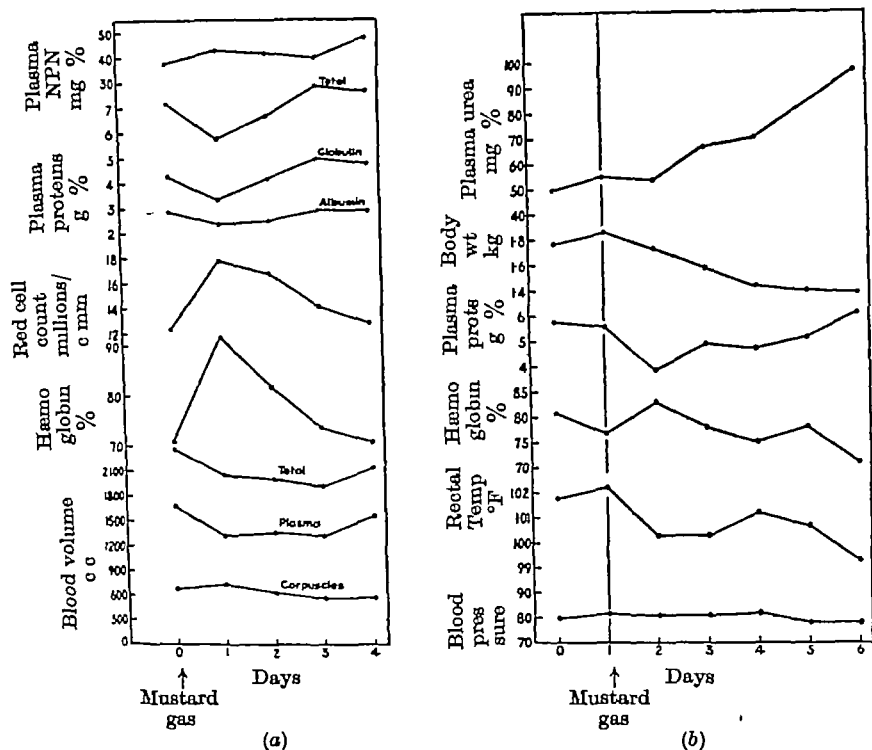


FIG. 3—The effects of skin contamination with mustard gas

- (a) Goats 40 mg/kg applied to clipped skin of back Mean of 3 experiments  
 (b) Rabbits 15 mg/kg applied to clipped skin of back Mean of 6 experiments

ear technique, did not fall in the experiments represented in fig 3. The rectal temperature, however, fell considerably.

Thus in the first phase of mustard-gas poisoning due to skin contamination with the liquid, a protein-rich fluid resembling plasma in composition leaks out of the circulation locally. The period of hæmoconcentration in animals lasts for only 1 to 2 days, and may be followed by a more prolonged phase during which the animal gradually becomes weaker and finally dies in about 6 to 10 days. During this time diarrhœa is generally observed, and profuse salivation is some-

times evident. The plasma protein concentration has by this time risen to normal. There is an increased secretion of fluid with a total nitrogen content of only 50 mg per cent into the alimentary canal. This phenomenon is apparent in the acute experiments in cats of Foss [1943], and in chronic experiments in dogs with fistulae carried out by Gregory [1943]. Thus in this second phase of fluid loss there is no hypoproteinæmia.

*Loss of Body-weight*—A striking feature of the behaviour of animals after the skin application of mustard-gas is the rapid loss of weight

TABLE I—THE TOTAL CIRCULATING PLASMA PROTEINS, GRAMS, BEFORE AND AFTER MUSTARD GAS CONTAMINATION

		Before mustard gas	After mustard gas Days			
			1	2	3	4
Mean of 3 goats	Total	94	66	76	91	96
	Albumin	37	28	29	33	36
	Globulin	57	38	47	58	60
Mean of 4 rabbits	Total	6.5	4.4	5.6	6.0	6.2
	Albumin	3.6	2.5	2.9	3.1	2.8
	Globulin	2.9	1.9	2.7	2.9	3.4

with resultant emaciation during the first week. Fig. 4 depicts the fall in body-weight in rabbits and guinea-pigs after skin application and subcutaneous injection of mustard-gas. The weight of groups of controls that were given the same amount of food remained constant over a given period. The contaminated animals, however, did not eat all the food given them each day.

In attempting to discover the cause of wasting, loss of appetite and fasting must be considered. After mustard-gas contamination, animals may appear ill for several days, whilst it is a frequent observation that gastro-intestinal lesions develop which might cause anorexia and wasting. The amount of food eaten by rabbits after skin contamination with 5 and 10 mg mustard-gas per kg body-weight was investigated. The rabbits were given a known quantity of food each day before and after mustard-gas application, and the proportion eaten was determined by measuring the amount left each morning. There were 10 rabbits in each group. With a dose of 10 mg/kg, there follows a period of 4 days during which less food is taken, the mean percentage of food eaten falling to 58 on the second and third days. With 5 mg/kg the



mean percentage of food eaten falls to 83 on the third day. With larger doses the animals eat less, and salivation and severe diarrhoea are often observed. Although a decreased food intake is one of the causes of loss of body-weight, the loss of fluid by diarrhoea and salivation is also a contributing factor. For example, in rabbits which are given

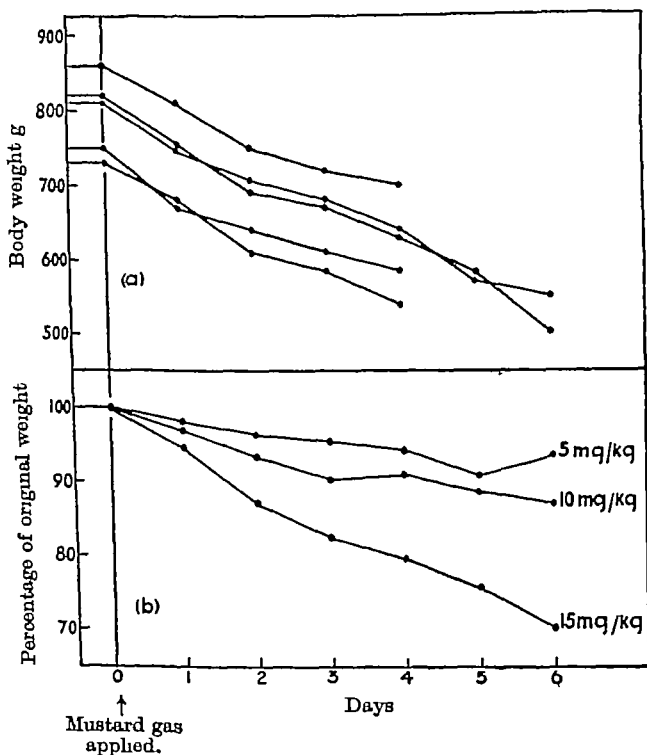


FIG 4—The effects of mustard gas on body weight

- (a) Guinea pigs Subcutaneous injection of 10 mg/kg mustard gas diluted in tributyrin  
 (b) Rabbits Skin application of mustard gas Mean of 10 experiments for each dose

no food for 5 days, animals contaminated with mustard-gas lose more weight than control animals (fig 6 (b))

*The Water Content of the Body Tissues*—The tissues of many animals dying from mustard-gas poisoning appear to be dehydrated. In order to investigate this dehydration, the water content of various tissues was determined in guinea-pigs after the subcutaneous administration of 10 mg mustard-gas per kg body-weight. Twenty guinea-pigs were so injected. Seven were killed on the fourth day, 7 on the fifth, and 6 on the sixth. The water content of the muscle, lungs, small intestine, and skin of these 20 animals and of 20 normal animals was

determined The muscle, intestine, and skin were taken from the same position in each animal, while the water content of the whole

TABLE II — THE WATER CONTENT OF VARIOUS TISSUES OF GUINEA PIGS BEFORE AND AFTER SUBCUTANEOUS INJECTION OF 10 MG/KG MUSTARD GAS DILUTED IN TRIBUTYRIN

	Mean of 20 controls	Mean of 20 controls after mustard gas	P
Muscle	73.85 $\pm$ 0.19	74.34 $\pm$ 0.80	0.5-0.6
Skin	65.33 $\pm$ 0.49	60.56 $\pm$ 0.45	<0.00003
Lungs	78.17 $\pm$ 0.31	75.55 $\pm$ 0.50	<0.0001
Intestine	80.11 $\pm$ 0.20	84.75 $\pm$ 0.57	<0.00003

lungs was estimated in each case The skin was taken from the back, well away from the site of injection, since locally the skin was very

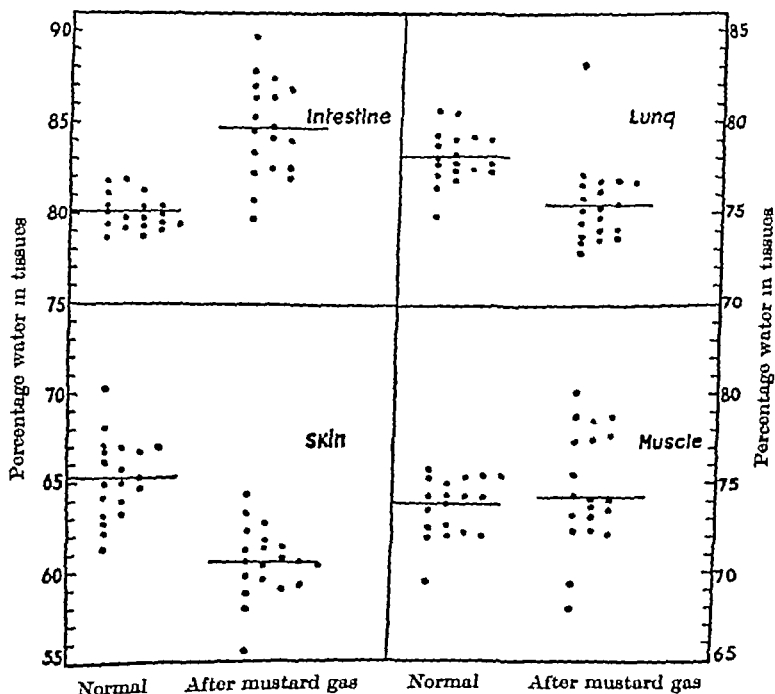


FIG 5 — The water content of the skin, intestines, lungs, and muscles of guinea pigs before and after subcutaneous injection of 10 mg/kg mustard gas

oedematous The method of water estimation used was a modification of the method of Holt and Callow [1941] The average results are given in Table II and individual results plotted in fig 5 The muscles showed

no significant alteration in water content, while the skin and lungs contained less water. The intestine was swollen and cedematous, and contained more water than in the control animals.

Thus it seems that when diarrhoea develops as a result of mustard-gas poisoning, water is lost from the bowel causing dehydration in some tissues. The actual percentage of water of the muscle in these experiments did not fall, but it is probable that muscle is broken down to supply water, as will be seen in the next section.

TABLE III—THE PLASMA N.P.N. MG PER CENT BEFORE AND AFTER SKIN APPLICATION OF MUSTARD GAS, 15 MG/KG, IN RABBITS

	Days before mustard gas		Days after mustard gas									
	1	0	1	2	3	4	5	6	7			
1	39	40	50	55	74	Died	Killed					
2	46	34	53	60	50	53						
3	46	37	35	45	403	Died						
4	34	25	30	27	46	Died	Killed	137 69	Died 46 Killed			
5	41	47	134	282	Died	31						
6	30	28	31	32	31							
7	31	28	40	40	47	Died Died	84 56	126 64 Died Killed				
8	38	25	38	35	36		200 59					
9	30	30	42	40	49							
10	42	36	42	67	158	54 38 128 46	Died Died					
11	42	35	58	165	Died							
12	36	30	34	36	55							
13	30	28	40	32	36	105 39	Killed					
14	26	31	52	64	105							
15	26	29	49	36	39							

### *Effects on Renal Function and Nitrogen Metabolism*

(1) *Chronic Experiments in Rabbits*—In early experiments it was noticed that coincident with body wasting the plasma N.P.N. rose. The changes observed in the plasma N.P.N. in 15 rabbits after the skin application of 15 mg mustard-gas per kg body-weight are given in Table III. The increase in many cases is pronounced. Experiments were, therefore, performed to investigate the nitrogenous metabolism following the skin application of mustard-gas. The daily urea output and the plasma urea were determined, the rabbits being kept in metabolism cages and catheterized each morning.

A series of 6 rabbits was first investigated in this way, the animals being given food and water *ad lib*. The results of a typical experiment

determined The muscle, intestine, and skin were taken from the same position in each animal, while the water content of the whole

TABLE II—THE WATER CONTENT OF VARIOUS TISSUES OF GUINEA PIGS BEFORE AND AFTER SUBCUTANEOUS INJECTION OF 10 MG /KG MUSTARD GAS DILUTED IN TRIBUTYRIN

	Mean of 20 controls	Mean of 20 controls after mustard gas	P
Muscle	73.85 $\pm$ 0.19	74.34 $\pm$ 0.80	0.5-0.6
Skin	65.33 $\pm$ 0.49	60.56 $\pm$ 0.45	<0.00003
Lungs	78.17 $\pm$ 0.31	75.55 $\pm$ 0.50	<0.0001
Intestine	80.11 $\pm$ 0.20	84.75 $\pm$ 0.57	<0.00003

lungs was estimated in each case The skin was taken from the back, well away from the site of injection, since locally the skin was very

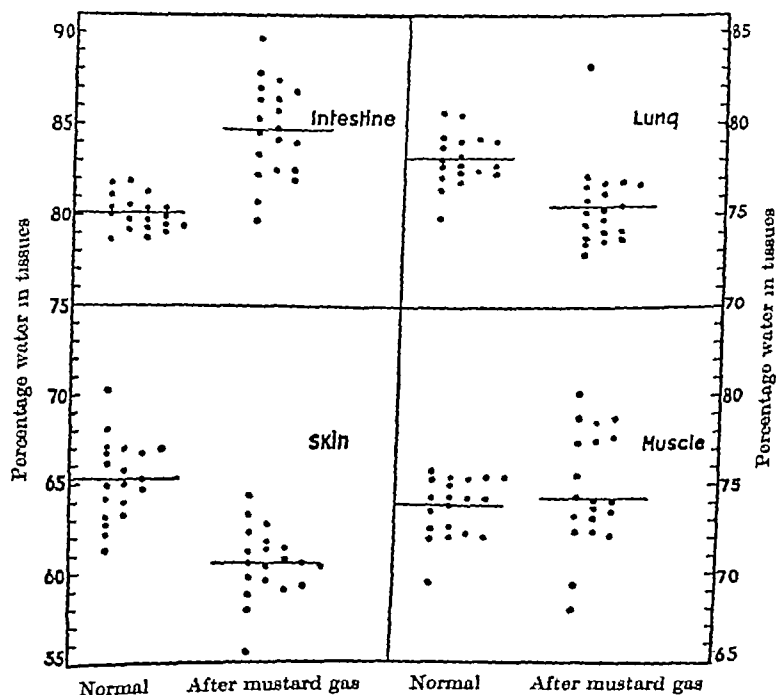


FIG 5—The water content of the skin, intestines, lungs, and muscles of guinea pigs before and after subcutaneous injection of 10 mg /kg mustard gas

œdematous The method of water estimation used was a modification of the method of Holt and Callow [1941] The average results are given in Table II and individual results plotted in fig 5 The muscles showed

mustard-gas in high doses, further experiments were performed in which food was withheld but water given *ad lib* for 5 days after application and for a similar period in control animals. The results of one such series are shown in fig 6 (b). The body-weight falls more in the

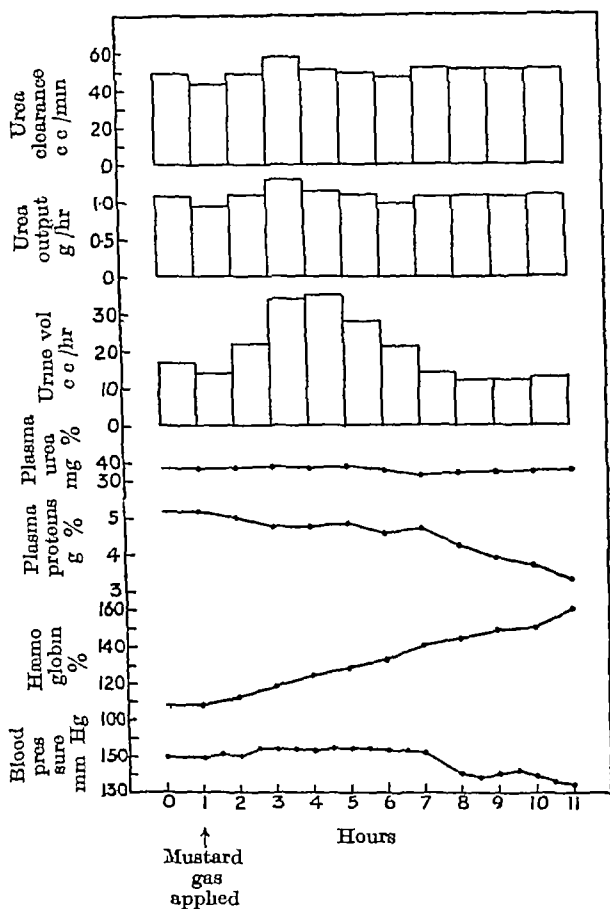


FIG 7—The changes in blood and urine induced by skin application of 40 mg/kg mustard gas in a dog anaesthetized with sodium barbitone

contaminated animals than in the controls, and associated with this increased fall in weight is a rise in urea output and in plasma urea.

All animals do not have diarrhoea after mustard-gas contamination, but in those that do, wasting, oliguria, increased plasma urea, and increased urea output are generally observed.

(u) *Acute Experiments in Anaesthetized Dogs*—The renal function has been studied in acute experiments where haemoconcentration has been produced in dogs by the skin application of mustard-gas. These acute experiments cannot show the effects of the prolonged dehydra-

are shown in fig 6 (a) After the application of mustard gas to the skin, the urine volume falls and becomes fairly constant at about 50 c c per day The concentration of urea in the urine rises, often to 6 per cent The urea output per day rises, often to twice the normal,

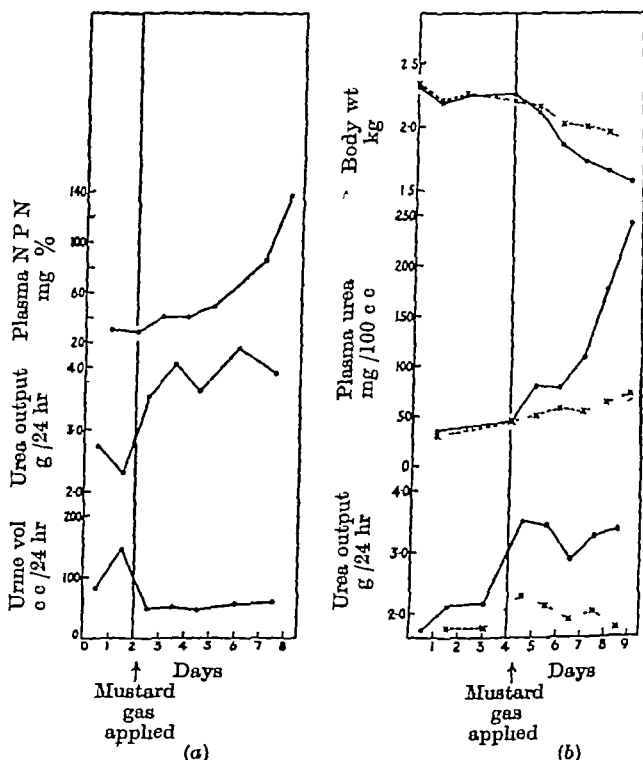


Fig 6—The changes in plasma and urine urea after skin contamination with mustard gas, 15 mg/kg, in rabbits

(a) Food and water *ad lib*

(b) Food withheld for 5 days after mustard gas and for similar period in controls, but water *ad lib*

× — × Mean of 2 controls  
● — ● Mean of 2 contaminated

and associated with this increase in the output of urea is a rise in the plasma urea, gradual at first but more rapid as the animal becomes weaker. Thus in spite of the fact that more urea than normal is being excreted, a great deal is accumulating in the tissues, so the actual breakdown of protein must be greatly increased. Since the intake of food by these rabbits was much less than before the application of mustard-gas, the protein breakdown must have been mainly endogenous, representing a destruction of body tissue. The body-weight of the rabbit represented in fig 6 (a) fell from 2.35 to 1.42 kg in 6 days.

Since most of the rabbits eat very little after skin application of

1 g urea per kg body-weight was injected intravenously into a normal dog, while in fig 8 (b) the same amount of urea was injected into a dog 6 hours after skin contamination with mustard-gas when the blood was considerably concentrated. In the first dog the plasma urea rose from 51 to 190 mg per cent 5 minutes after injection and fell to 80 mg per cent in 4 hours. This fall in 4 hours was associated with considerable diuresis and increased urea output. In the second dog the plasma urea rose from 60 to 206 mg per cent 5 minutes after injection and fell to only 160 mg per cent in 4 hours, and the diuresis was less marked and of a much shorter duration. In the normal dog 75 per cent of the excess urea was excreted, while in the mustard-gas contaminated dog only 25 per cent of the excess urea was excreted in 4 hours. In both, the blood-pressure showed a slight rise after the injection of the urea.

These experiments indicate that the kidneys in cases of anhydræmia are less able to get rid of an excess formation of urea. Either the filtration rate is less in the dehydrated dog or the reabsorption of both water and urea is increased. Thus it seems that in anhydræmia if the urea production is increased, the urea output may be increased, but the plasma urea will also increase.

### DISCUSSION

Mustard-gas affects the skin whether applied as a vapour or liquid. This investigation deals only with liquid contamination. Two distinct phases in the disturbance of water balance in animals as a result of heavy skin contamination with liquid mustard-gas may be observed. The first is caused by the local action on the capillary membrane, increasing the permeability to plasma proteins. The local loss of plasma into the skin may cause a decreased plasma volume, concentration of the blood, and hypoproteinæmia. The onset of these signs depends upon the rapidity with which the mustard-gas penetrates the skin, and upon the balance between the rates of outgoing fluid locally and reabsorption of fluid from the unaffected tissues. It has been noted in dogs that the rapidity with which mustard-gas penetrates the skin and produces cedema may vary considerably in different animals, for the onset of increased lymph flow may be very gradual or very quick. In rabbits, the reabsorption of fluid seems to progress at a more rapid rate than in dogs and goats, so that the degree of hæmoconcentration is not very great in the former although the plasma protein level may fall considerably.

In man, the penetration of small amounts of mustard-gas is normally fairly rapid, but is much greater in a hot, sweaty skin than in a cold skin [Cullumbine, 1947]. In the experimental animals used here, where there are no sweat glands present in the areas of skin contaminated,

tion, but give information regarding the early stages where plasma is being lost through the damaged capillary membranes. Dogs were anaesthetized with sodium barbitone and urine collected by passing a catheter through the urethra into the bladder.

In fig 7 the effects of mustard-gas, 40 mg per kg, applied to the

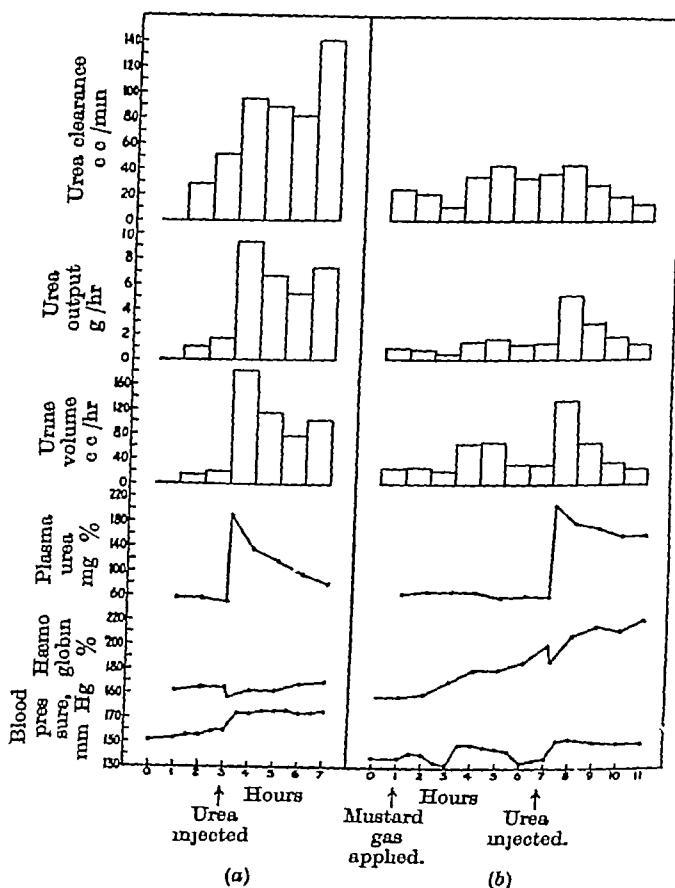


FIG 8—The changes in blood and urine following injection of 1 g/kg urea intravenously in  
(a) Normal dog anaesthetized with sodium barbitone,  
(b) Dog anaesthetized with sodium barbitone and 6 hours after skin application of 40 mg/kg mustard gas

skin of the abdomen are shown. As oedema is produced the blood concentrates, but vasoconstriction prevents a fall in blood pressure, recorded from a carotid artery. The urea output, urea clearance, and plasma urea remain constant. In these experiments insufficient time has elapsed for any noticeable changes in nitrogen metabolism to occur.

The behaviour of the kidney during haemoconcentration when the urea production is suddenly increased is shown in fig 8. In fig 8 (a),



that by keeping the contaminated limbs cold as in similar experiments with thermal burns [Courtice, 1946], the local œdema was greatly reduced in rats and rabbits, but again the ultimate mortality rate was not decreased. These experiments indicate that the second phase in mustard-gas poisoning is probably more important than the first.

In the second phase, the fluid loss from the gut does not lead to hypoproteinæmia. This indicates the replacement of fluid and electrolytes. Courtice was unable to show any benefit from glucose or glucose-saline injections, but Cullumbine reduced the mortality somewhat by the administration of sodium chloride solutions by mouth, subcutaneously or intraperitoneally if the treatment was begun early [Cullumbine, 1947].

It must be remembered, however, that besides the effects on water-balance, mustard-gas may have a considerable effect on the blood-forming tissues. Thus even though the water-balance may be restored by treatment, the white cells in the blood may be greatly reduced or may almost disappear altogether. All attempts to prevent the bone-marrow and lymph gland changes in mustard-gas poisoning have so far failed.

### SUMMARY

The lymph flow from the skin of dogs contaminated with mustard-gas increases, and the lymph protein concentration approaches that in the plasma.

The lymph taken after contamination inhibits the growth of bone-marrow fragments in tissue culture, indicating the presence of mustard-gas or a toxic derivative.

The effect of local œdema is to produce hæmoconcentration and hypoproteinæmia lasting 1 to 2 days in goats and rabbits.

The absorption of mustard-gas leads often to diarrhœa, with loss of fluid from the bowel. This causes dehydration without hypoproteinæmia. The dehydration results in loss of body-weight, increased protein breakdown, and rise in blood urea.

We are indebted to Mr F W Randall for his assistance in the experiments with tissue culture. Our acknowledgments are also due to the Chief Scientist, Ministry of Supply, for permission to publish this investigation.

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this factor does not enter, but it seems that other factors may vary the rate of penetration of the mustard-gas

The second phase may overlap the first. It is due to the absorption of mustard-gas and its action on the alimentary canal leading to diarrhoea in the contaminated animals. Evidence has been given which suggests that some absorption may take place via the lymphatics. Although mustard-gas combines with proteins, and although the tissue fluid contains some protein, this does not necessarily mean that all the mustard-gas absorbed combines with these proteins before it enters the blood-stream. If it did, then it should be absorbed entirely by the lymphatics, so rendering absorption a slow process. Although some of the mustard-gas may be absorbed by the lymphatics of the skin, it seems probable that most of it enters the blood capillaries directly. The fact that none was detected in the blood in the experiments described above, may be due to the relatively large flow of blood through a limb compared with the lymph flow.

The excretion of some of this absorbed mustard-gas by the liver may be the cause of the alimentary symptoms. Boursnell, Cohen, Dixon, Francis, Greville, Needham, and Wormall [1946] have shown that mustard-gas, containing  $S^{35}$  as tracer element, injected intravenously, is excreted in the urine and also in the bile. The effect on the gut of skin contamination may vary considerably in different animals of the same species. It is possible that this variation may depend partly on the rate of absorption from the skin, and partly on the rate of excretion by the liver in the bile. Thus in a group of animals contaminated in the same way, some may show little or no alimentary effects, whereas in others severe diarrhoea is evident.

In those animals that do show severe diarrhoea, dehydration and rapid wasting ensue, generally leading to death. Associated with this dehydration and wasting, the signs of oliguria, increased protein breakdown and raised blood urea are observed. This syndrome is characteristic of dehydration from many causes, and has already been discussed by Cameron, Courtice, and Short [1947] when describing lewisite poisoning. The inability of the kidney to prevent a rise of blood urea appears to be due to a circulatory change. There may be vasoconstriction of the renal vessels, thus decreasing the filtration rate, or the circulatory changes described by Trueta, Barclay, Daniel, Franklin, and Prichard [1947] in rabbits in ischaemic shock may account for the changes observed.

Thus in treatment of severe mustard-gas poisoning several factors have to be considered. Experiments have been performed in which the local oedema was reduced. Cameron [1945] applied pressure bandages to the legs of goats contaminated with mustard-gas. The local oedema and the degree of haemoconcentration were greatly reduced, but the mortality rate did not decrease. Courtice (unpublished data) showed

A GAS-ANALYSIS APPARATUS FOR 200 c mm SAMPLES By  
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IN the course of a series of experiments on the composition of respiratory and of pneumothorax gases in rabbits, fifty or more small samples had to be analysed in succession. For this a method was required which combined a fair accuracy with simple handling, easy cleaning, and great reliability. The method finally adopted is principally based on the work of Schmit-Jensen [1920]. A number of modifications have, however, been incorporated to meet the specified requirements.

*Principle*—The apparatus is of the constant-pressure type originally introduced by Timiriazeff [1877, 1885] and later greatly improved by Krogh [1908]. It essentially consists of a long, graduated capillary glass tube in which the length of a gas bubble is measured before and after the absorption. In the apparatus of Krogh and in that of Schmit-Jensen, as indeed in most micro-gas analysis apparatus, the absorption is effected in the open funnel at the end of the burette. When, however, the size of the gas bubble is increased to 150–200 c mm this is no longer feasible on account of the large size of the funnel and the resulting danger of the liquid dropping out. Therefore the absorption has been carried out in separate absorption pipettes which, when required, could be attached to the burette.

Both the fluid in the burette and the absorbing reagents are watery solutions, and thus an error is introduced in the readings due to the solubility of gases. Many investigators have tried to reduce the error by the use of mercury. This, however, is found very soon to contaminate the inner surface of the narrow capillary tube. In recent times Scholander and his associates [1942, 1947] solved this problem by using a micrometer burette filled with mercury. The volume of the gas bubble is no longer read from a graduated glass tube but from the scale of the micrometer. On the other hand, in using watery solutions throughout a fair degree of accuracy may also be attained by taking some precautions for keeping the systematic error sufficiently constant and by applying the proper corrections. This method permits a very simple construction of the apparatus and thereby promotes the ease of

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as it never has to contain the whole gas bubble either during the introduction of the gas or during the absorption. The funnel *a* at the other end of the graduated tube is of larger size (inside dimensions  $6 \times 40$  mm), in order to accommodate a piston and to offer sufficient grip to the piston-housing. The second capillary tube (*b*), which serves as a thermobarometer, has only to be graduated over a short distance, and is open to the atmospheric air at one end, but at the other end tightly stoppered by a solid rubber stopper as soon as a gas bubble of suitable size has been introduced.

The air-tight enclosure of the piston (fig 2, *c*) is ensured by a ring of packing material pressed down by the nut *e*. The complete piston-housing may be readily removed in order to facilitate the cleaning of the graduated glass tube. Three stiff rubber rings (*b*) turned on a lathe

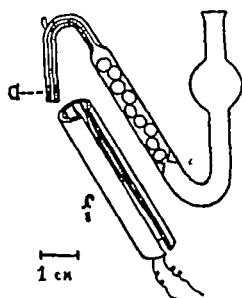


FIG 3 —Absorption vessel and heating jacket

to fit over the larger funnel *a* of the capillary are compressed by turning the nut *f* and in this way a rigid and air-tight connexion is realised.

At the beginning of an analysis the burette is filled with fluid. As at a higher rate more liquid will be left on the wall of the tube and the actual diameter of the gas bubble will decrease, the rate at which the gas is sucked in has to be controlled. Moreover, gas is dissolving in this layer of fluid and the amount will depend on the thickness of the film. In order to minimise the resulting accidental error the gas bubble has to be sucked in at a constant rate, as has already been pointed out by Schmit-Jensen. The use of a smooth piston enabled us to replace the clockwork mechanism used by this author by a more compact unit. An automatic release mechanism (*d*) as used in photography has been supplied with an extra support (*g*) for attachment to the piston-housing. The movable grip with which the mechanism is provided has been made to pull at the piston, and so the gas bubble could be pulled into the tube at a uniform rate. The speed of the clockwork mechanism has to be regulated so that the velocity of the fluid meniscus does not exceed 10 cm in 15 seconds.

The reagents for the absorption of  $\text{CO}_2$  and  $\text{O}_2$  are contained in vessels (fig 3) which may be fastened to the graduated burette by

dismounting and of cleaning This is of prime importance, as absolute cleanliness is a chief requisite in analysis of this kind

*Apparatus*—Two capillary glass tubes being selected for uniform diameter are surrounded by a water-jacket (fig 1) One of these (a-e)

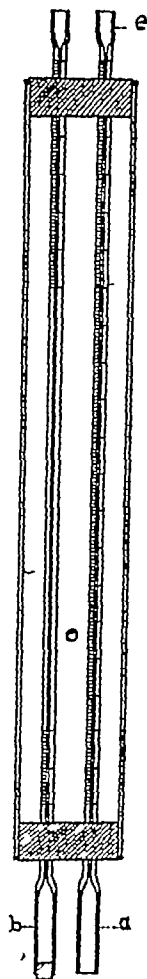


FIG 1—Gas analysis apparatus in water jacket (see text)

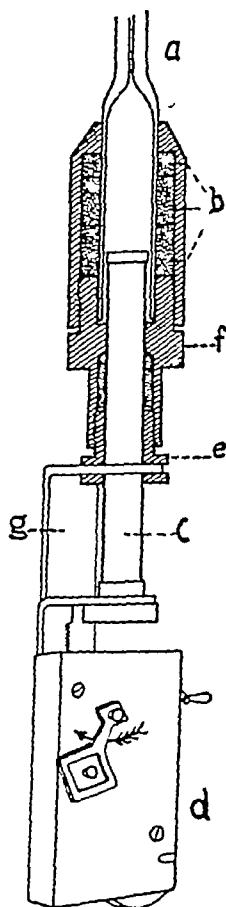


FIG 2—Fixation of piston to funnel a clockwork attached

is graduated in 200 divisions over a length of 30 cm, the zero-point lying outside the water-jacket at about 5 mm from the top of funnel e. The internal diameter being about 1 mm, the capacity of the dry burette is about 240 c mm. When moistened this is reduced to about 200 c mm. Notwithstanding the relatively large size of the gas bubbles to be analysed the funnel e may be small (inside dimensions 5 × 17 mm),

advantage both by the ease with which complete absorption was reached and by the decrease of viscosity of the reagent. Heating to  $25^{\circ}$ – $30^{\circ}$  C was readily accomplished by a 2-V battery and a piece of wire of 50 cm length having a resistance of  $14\ \Omega$  which was mounted lengthwise on

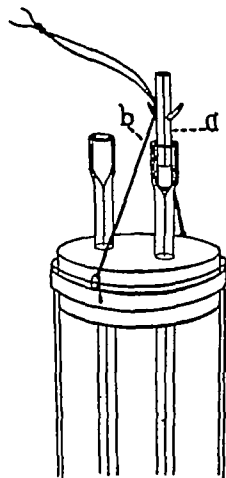


FIG. 4 —Glass tube fixed with rubber strap in the funnel of the capillary

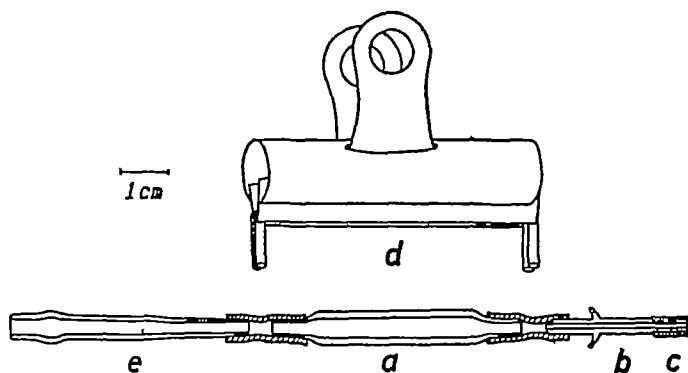


FIG. 5 —Sampling tube and clamp

the inside of a piece of rubber tubing fitting on the absorption tube (fig 3, f)

*Transfer of the Gas Sample* —To avoid the disappearance of gas by diffusion to the G S mixture in the funnel when a sample is introduced in the burette, a small glass tube has been employed (fig 4, a, fig 5, b). This tube (length 3 cm, inside diam 0.8 mm, outside diam 3 mm) has been ground off at the tip for about 5 mm to accommodate a short

means of a rubber strap without any need of a supplementary support (cf fig 4) The tip of the capillary part of the absorption vessel (fig 3, a) has been ground to accommodate a short piece of rubber tubing The elastic strap presses this down into the funnel and in this simple way a gas- and water-tight joint is obtained The bore of the capillary part has to approach 0.35 mm, a wider bore unduly increasing the dead space and a finer one causing the ready formation of small drops of reagent which may contaminate the burette The transition from the capillary to the wide part of the absorption vessel is to be made as abrupt as possible in order to break any drops which may have been formed For the same reason a small space has to be left between the end of the capillary and the upper glass bead of the series which fills up part of the tube to enlarge the absorbing surface

Trouble-free absorption can only be ensured when clean pipettes are used Therefore it has been our custom to clean the tubes and to charge them freshly with reagents after every fifteen analyses This may readily be done by connecting the tube to a filter-pump, rinsing it with water and afterwards drying it with a small quantity of alcohol, which, however, has to be thoroughly removed

*Reagents* — In order to reduce as much as possible the error due to the solubility of gases, Schmit-Jensen replaced the acidulated water with which Krogh originally filled his apparatus by a mixture of equal parts of glycerol and saturated NaCl solution acidulated with sulphuric acid The viscosity of this fluid, however, is rather high, especially on a cold day As this causes a rise of the accidental error, it has been our custom to change over to a glycerine-salt (G S) mixture of 45 vol per cent, when the room temperature dropped to about 17° C The mean viscosity (relative to water of 17.4° C) of the 45 per cent mixture between 12° and 17° C about equals that of the 50 per cent mixture from 17° to 22°

Initially a 10 per cent KOH solution was employed for the absorption of the CO<sub>2</sub> Although the solubility of O<sub>2</sub> and N<sub>2</sub> is known to be small, the repeated contact of the gas bubble with the relatively large quantity of diluted solution causes an error which cannot be neglected, especially when the O<sub>2</sub> and N<sub>2</sub> content of the sample differs markedly from that of the atmospheric air with which the KOH is in equilibrium Therefore later on a more concentrated KOH solution (33 per cent) has been used, but even then the error was not completely abolished as will be shown later on The use of more concentrated lye solutions proved to be impracticable on account of their high viscosity

The oxygen was absorbed by pyrogallol acid (10 g pyrogallol acid in 100 g KOH solution of sp gr 1.52), kept from contact with the air by a layer of fluid paraffin Heating of the reagent was found to be necessary when the room temperature dropped to about 15° C, but also above this temperature the heating turned out to be of great



capillary part and to break at its mouth, thereby contaminating the graduated tube. This may often be prevented by a timely return of the gas to the absorption pipette, the drops break at the other end and a second trial may prove to be more successful. Cleaning of the vessel, however, is indicated.

After disconnecting the potash pipette the second reading is taken, with the apparatus again in the horizontal position. The pyrogallate pipette is now fixed and the bubble moved to and fro during two minutes, which time commonly suffices for complete absorption. On reintroducing the gas for the final measurement a small column of reagent will follow the bubble. As the opaque fluid obscures the curvature of the meniscus it has to be removed. After disconnection of the absorption vessel this is accomplished by filling the funnel with acidulated water or G S mixture and turning the apparatus upside-down. The heavy pyrogallate is then seen to drop bodily through the surrounding fluid. The meniscus being cleared, the apparatus is brought back to the horizontal position and the third and final pair of readings are made. The funnel and the upper part of the graduated tube are thoroughly washed with acidulated water, the residual gas and part of the G S mixture are expelled by pressing the plunger hard down and by emptying the funnel, after which the expelled fluid is replaced by a quantity of fresh G S mixture.

Notwithstanding all precautions the burette gradually gets dirty and therefore has to be cleaned after every ten to fifteen analyses. This may readily be performed by removing the plunger-housing and rinsing the whole with bichromate of potash and with water. It has been our custom to clean and freshly to charge the absorption vessels at the same time. A complete analysis of both  $\text{CO}_2$  and  $\text{O}_2$  may be conducted in ten minutes.

*Corrections* —(a) Changes in temperature and barometric pressure are corrected by means of the readings of the thermo-barometer.

(b) As a gas bubble in a capillary tube has spherical ends, its measured length has to be corrected for the curvature of the menisci. The internal diameter of the burette being 1 mm, the readings have to be reduced by 0.3 mm [Schmit-Jensen].

(c) On account of its high solubility an appreciable amount of  $\text{CO}_2$  is lost to the layer of G S mixture adhering to the inner wall of the burette before the first reading is made. The resulting systematic error is not exactly proportional to the concentration of  $\text{CO}_2$  present.

If a volume of gas ( $V$ ) containing  $z$  per cent  $\text{CO}_2$  is introduced into the burette, a constant fraction, say  $a$ , of the amount of  $\text{CO}_2$  is lost to the film of G S mixture. As  $\frac{az}{100}V$  dissolves, the first reading is  $V\left(1 - \frac{az}{100}\right)$ .

piece of rubber tubing (fig 5, c) By pressing this tube down into the funnel by means of a rubber strap (fig 4, b) fastened to the water jacket of the apparatus, a gas- and water-tight connexion is obtained

By a short piece of rubber tubing the capillary (fig 5, b) is joined to the sample vessel *a*, which may be closed at both ends by a clamp (*d*) The filling of the sample vessel may either be done by flushing when enough gas is available, or by first filling the sample vessel and the capillary with mercury and then emptying both through tube *e* while the gas to be analysed is flowing in through the capillary Before connecting the capillary to the burette in the manner shown in fig 4, both capillary *b* and tube *e* are filled with G S mixture from a syringe fitted with a long thin needle In doing this, care must be taken to prevent small air bubbles getting entrapped

*Procedure* —As absolute cleanliness of the apparatus is essential for obtaining uniform results, the graduated capillary and the absorption vessels are kept filled with potassium-bichromate solution when not in use With a new burette it sometimes takes weeks to attain a degree of cleanliness sufficient for the conduction of faultless analyses After rinsing the burette with water at the filter-pump the apparatus is reassembled and filled with G S mixture One of the absorption pipettes is charged with KOH solution, and another with pyrogallie acid from a stock solution stored over mercury Before the introduction of the sample the G S is raised to half fill the funnel of the apparatus

Both ends of the sample vessel being filled with the G S mixture up to the clamp, the capillary tube is now fixed to the burette by means of the rubber strap After removal of the clamp the lower meniscus of the gas bubble is cautiously moved downwards by turning the piston by hand When the meniscus is about 2 mm beneath the mouth of the capillary tube, the clockwork mechanism is coupled to the apparatus and a gas bubble sucked in at uniform speed When a sufficient quantity of gas has been introduced, first the clockwork and then the capillary tube is disconnected The apparatus is turned in a horizontal position, and by careful movement of the piston the meniscus is brought to zero The first reading is taken, both of the burette and the thermo-barometer The apparatus being returned to the vertical position, the upper meniscus is raised to the bottom of the funnel so that the mouth of the potash pipette, which is now placed on top, may reach the surface of the bubble In this way the contamination of the reagent by the G S in the funnel is negligible The transfer of the gas is executed very slowly at first so as to leave but a very thin film of fluid in the capillary part of the absorption vessel The bubble is moved six times to and fro between potash pipette and graduated capillary, the final return being accomplished by the clockwork mechanism When the absorption tube is getting dirty by frequent use, small drops of reagent may be seen to form themselves in the

on introducing the gas into the burette, contact of the bubble with a large quantity of fluid is avoided

The value of  $a$  has to be determined for each apparatus by analysing gas mixtures of known  $\text{CO}_2$  content. These mixtures have been prepared by weighing the mercury running from a vessel in which it was replaced by the calculated quantities of  $\text{CO}_2$  or air. On attending to the proper precautions, the resulting mixture may be accurate to 0.05 per cent. By subtracting the apparent percentage of  $\text{CO}_2$ , as found by analysis, from the true one resulting from the weighing, the systematic error is determined. On application of this value to the formula given above ( $\text{syst. error} = az \left(1 - \frac{z}{100}\right)$  per cent) the value of  $a$  may be calculated. An example of the determination of the system-

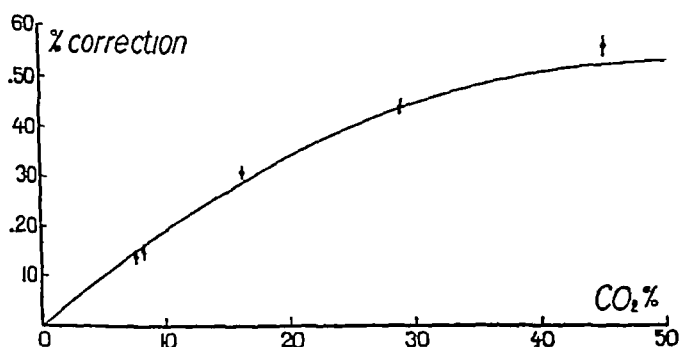


FIG. 6—Systematical error (correction) caused by the solution of carbon dioxide in the confining liquid, plotted against the true  $\text{CO}_2$  percentage

atic error and the factor  $a$  is presented in Table I, while in fig. 6 the systematic error has been plotted against the concentration of  $\text{CO}_2$  in the gas mixture. The correction to be applied to the apparent  $\text{CO}_2$  concentration may be read from this diagram.

Moreover, the solubility of the  $\text{CO}_2$  not only causes a systematic error in the determination of the  $\text{CO}_2$ , but also of the oxygen present in the gas sample, as the initial reading gives not the real volume  $V$  but  $V \left(1 - \frac{az}{100}\right)$ . Therefore  $\frac{az}{100}V$  has to be added to the apparent volume of the gas bubble in order to calculate the real content of these gases. At a  $\text{CO}_2$  content of about 5 per cent commonly met with in biological experiments, this correction, when  $a = 0.02$ , amounts to 0.02 of 5 per cent, i.e. to 0.1 per cent of the initial volume. This means that with a bubble of 30 cm length a correction has to be applied of 0.3 mm, being of the same size but of opposite sign as the meniscus correction.

(d) The solubility of  $\text{O}_2$  and  $\text{N}_2$  being much smaller than that of

The amount of  $\text{CO}_2$  absorbed by the KOH between the first and the second reading amounts to  $\frac{z}{100}(1-a)V$

Thus the apparent  $\text{CO}_2$  content of the gas sample is  $\frac{z(1-a)}{100-az}$  100 per cent

The systematic error, i.e. the difference between the true and the apparent  $\text{CO}_2$  content, amounts to  $\frac{az(100-z)}{100-az}$  per cent

TABLE I

True composition Per cent $\text{CO}_2$	Apparent composition Per cent $\text{CO}_2$ Analytical results					Systematic error		
						Mean	Per cent	$a$
7.73	7.57	7.61	7.59	7.69	7.52	7.59	0.14	0.020
	7.55	7.53	7.62	7.63	7.62			
8.34	8.11	8.21	8.17	8.13	8.19	8.19	0.15	0.020
	8.22	8.26	8.15	8.25				
16.24	15.98	15.97	15.88	15.92		15.93	0.31	0.023
	15.97	15.98	15.94	15.87				
	15.97							
28.86	28.45	28.38	28.45	28.44		28.42	0.44	0.021
	28.38							
45.03	44.45	44.30	44.42	44.43		44.46	0.57	0.023
	44.50	44.51	44.50	44.42				
						Mean	0.021	

As  $az$  is always negligibly small with regard to 100, this may further be simplified to  $az\left(1 - \frac{z}{100}\right)$  per cent. Obviously the systematic error will become zero both for  $z=0$  and for  $z=100$ .

The value of  $a$ , i.e. of the factor determining the size of the systematic error at a given concentration of  $\text{CO}_2$ , depends on

(1) The solubility of carbon dioxide in the G.S. mixture

(2) The amount of liquid adhering to the inner wall of the burette. This may be kept constant by introducing the bubble at exactly the same speed throughout, and by control of the temperature at which the analyses are performed.

(3) The personal factor. The need for uniform conduction of the micro-gas analytical procedure has been stressed by Fr. Meyer [1935]. In the present method the personal factor is of minor importance, as

(e) The sources of systematic error to be encountered in the micro-analysis of gases may be met with by applying the proper corrections. Against an accidental error, however, the results can be safeguarded only by simplicity of technique, scrupulous cleanliness, and by uniformity of the manipulations. The analytical results reproduced in Table I give an impression of the size of the accidental error attained with the technique described in this paper. A deviation from the mean value exceeding 0.1 per cent has been seldom met with, the standard deviation commonly amounts to 0.05–0.08 volume per cent of the total gas present.

*Sampling of the Alveolar Air in Rabbits*—One of the main uses made of the present technique consisted in the serial analysis of the alveolar air of the rabbit. Krogh and Krogh [1910] introduced the method of drawing samples of alveolar air from the neighbourhood of the bifurcation at the end of an expiration. The principle of their method has been adopted though the details of the technique have been greatly modified. In the present method the glass capillary of the sampling tube (fig. 5, *b*) has been replaced by a long, narrow tube ending at the height of the bifurcation of the animal. Tube *e* was connected to a suction apparatus consisting of two bottles, one filled with water about 1 m. above the other. Air from the neighbourhood of the bifurcation was sucked away at a constant rate of 200 to 250 c.c./min. controllable by a differential manometer type of flow meter. At the end of an expiration both ends of the sample tube were closed simultaneously by the clamp *d*. As the volume of the tubing up to the sample vessel amounted to approximately 0.03 c.c., this space is swept at the given velocity of the gas stream within a fraction of a second. Thus the composition of the gas entrapped in the sample tube may be taken to equal that of the alveolar air. After replacing the long, narrow tube by the glass capillary *b* the gas sample was transferred to the analysis apparatus in the manner described above.

### SUMMARY

A volumetric apparatus is described for the analysis of 200 c mm of gas. Simple construction, easy cleaning and handling, and great reliability are the points stressed. The various corrections to be applied in analysis of this kind are fully discussed.

The apparatus handles directly samples containing from zero to 100 per cent absorbable gases. The standard deviation amounts to 0.05–0.08 vol. per cent. A complete analysis of  $\text{CO}_2$  and  $\text{O}_2$  requires about 10 min.

A method is described for drawing alveolar samples in rabbits.

$\text{CO}_2$ , the amount of these gases dissolving in the liquid layer adhering to the inner wall of the burette is negligible under any circumstances. An appreciable amount may, however, be acquired from or lost to the potash solution, as during the absorption the gas bubble is in contact with a relatively large quantity of fluid in equilibrium with the atmospheric air. This error has been reduced by employing a 33 per cent potash solution, but may only be fully obviated if the tension of the oxygen and nitrogen in the solution can be brought to the same level as that in the gas. This, however, is impracticable when, as in our experiments, gas mixtures of very divergent composition are to be analysed.

Obviously both the second and the third reading of the analysis

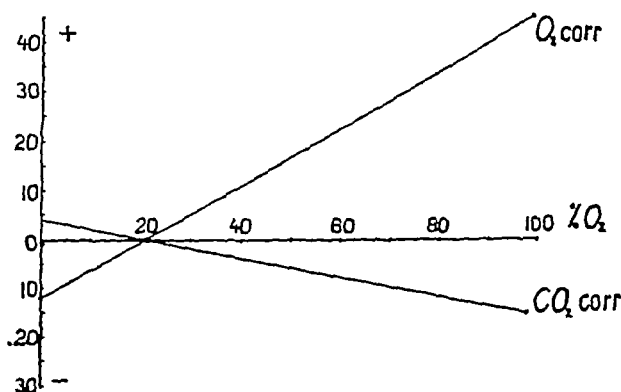


Fig. 7—Systematical errors (corrections) for  $\text{CO}_2$  and  $\text{O}_2$  caused by changes in the volume of  $\text{O}_2$  and  $\text{N}_2$  during the  $\text{CO}_2$  absorption

are affected by the error. From a gas mixture with a high oxygen content  $\text{O}_2$  is being taken up by the potash while at the same time  $\text{N}_2$  is given off. As the solubility of oxygen, however, is about twice as great as that of nitrogen, a quantity of gas is disappearing even when no  $\text{CO}_2$  is present in the sample. Moreover, the oxygen content as measured by the absorption with pyrogallate solution will be too low both on account of the loss of  $\text{O}_2$  to the potash and by the simultaneous increase of  $\text{N}_2$  in the bubble. On the other hand, when mixtures of low oxygen content are being analysed, the size of the bubble increases by the washing over the potash and the  $\text{O}_2$  content is found too high. In between at the atmospheric oxygen tension the systematic error is zero. Therefore with gas of an oxygen content below 21 per cent a correction for the solubility of  $\text{O}_2$  and  $\text{N}_2$  must be added to the apparent  $\text{CO}_2$  percentage and subtracted from the apparent  $\text{O}_2$  percentage, while the reverse has to be done when the oxygen content rises above 21 per cent. The diagram reproduced in fig. 7 has been composed from the results of the analysis of gases of known oxygen content.

# ALVEOLAR OXYGEN TENSION AND LUNG CIRCULATION

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## INTRODUCTION

ABOUT half a century ago various investigators, studying the existence of vasomotor influences on the lung blood-vessels, have mentioned a rise of pulmonary arterial pressure to occur in asphyxia, and have ascribed this effect to an increased resistance of the vascular bed of the lungs [reviewed by Tigerstedt, 1903] Emptying of the arterioles and capillaries of the lung during nitrogen respiration has been observed microscopally by Wearn *et al* [1926, 1934] These authors, however, regard this effect to be due to a general circulatory insufficiency, as a similar behaviour of the vessels was noted when death resulted from a failing circulation from any cause More recently a rise in pulmonary arterial pressure on breathing gases of low oxygen content has been described by von Euler and Lilljestr nd [1946] in cats, and by Motley, Cournaud, Werko, Himmelstein, and Dresdale [1947] in man In the opinion of the first-named authors, "the experiments seem to warrant the conclusion, that the regulation of the pulmonary blood-flow is mainly mediated by a local action of the blood and alveolar gases, leading to an adequate distribution of the blood through various parts of the lungs, according to the efficiency of aeration"

Although von Euler and Lilljestr nd by extirpation of the stellate ganglia, section of both vagi, and by artificial respiration have produced evidence that the rise of the pulmonary pressure is not due to nervous or mechanical factors, their statement cannot be accepted without a critical evaluation of other possibilities The breathing of gas of a low oxygen content by both lungs is bound to produce not only local anoxia, but general anox mia Therefore effects originating outside the lungs—*e.g.* changes in ventilation and circulation (heart, systemic blood-pressure, distribution of the blood), humoral (increased secretion of adrenaline) and nervous factors (stimulation of vasomotor centres)—must be taken into consideration

These disturbing influences are fully excluded in experiments on the isolated heart-lung preparation Experiments of this kind have been

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metal capillary tubes The amount of gas delivered by each capillary at a given pressure (1–8 atm) was determined in advance The various gases were made to flow together at a calculated rate to obtain the required mixtures The accuracy of this simple method proved to be sufficient for our purpose, as the composition of the mixtures kept constant within 1 per cent even in prolonged experiments in which the pressure in the storage flasks dropped from 150 to 10 atm

The composition of the alveolar air of the rabbits has been determined by a technique which is described elsewhere [Dirken and Heemstra, 1948] As sometimes fifty and more analyses had to be performed in succession, a simple and reliable micro-gas-analysis apparatus has been developed based on the method of Krogh [1908] and of Schmit-Jensen [1920]

*"Saturation Method"*—If one lung breathes a gas mixture of high and the other one a gas of low oxygen content, the oxygen saturation of the arterial blood of the systemic circulation will depend on the ratio of mixing of the blood from both lungs (In the present paper the terms "oxygen lung" and "nitrogen lung" will be used, indicating that the first one is being ventilated with gas of a higher oxygen content than the latter) The method seems to have been first adopted by Hess [1912], and its applicability is later discussed both by Moore and Cochran [1933] and by Jacobæus and Bruce [1940]

If in the course of an experiment the blood-flow through one of the lungs changes with respect to that of the other, thereby causing a change in mixing-ratio, the result will be a decrease or an increase of the saturation of the mixed arterial blood In the present series of experiments atmospheric air or gas of higher oxygen content has been administered continuously to the oxygen lung During an initial period the nitrogen lung was ventilated by an identical mixture, but was then switched over to a gas containing but little oxygen During the whole of the experimental period the oxygen saturation of the blood has been determined repeatedly The saturation has been measured by means of the photo-electric colorimeter constructed by Brinkman and Wildschut [1938], which requires no more than 0.2–0.3 c.c. blood For obtaining the blood sample the exposed carotid artery was compressed simultaneously in two places by the clamp depicted in fig. 1 and the vessel punctured in between On opening the clamp 0.35 c.c. blood was withdrawn from the artery during normal blood-flow After clamping the vessel and withdrawal of the needle, the puncture in the vascular wall was closed sideways by a *serre-fine* (fig. 2) In this manner a fairly good blood-flow was maintained in the artery during the interval between punctures, and the same puncture could be used repeatedly In order to prevent desiccation of the vascular wall and the formation of an intra-arterial thrombus, the carotid artery was carefully covered up each time by closing the wound

reported by Fuhner and Starling [1913], Löhner [1924], Drinker, Churchill, and Ferry [1926], but no reaction of the lung-vessels on ventilating the lungs with nitrogen has been observed. This negative evidence, however, is not to be regarded as conclusive on account of the abnormal conditions reigning in perfusion experiments—a point which has also been stressed by Wiggers [1921] and Hamilton [1944].

In the study of the local effect of hypoxia on the lung circulation in the intact animal, general anoxæmia is one of the principal sources of error to be prevented. There appears to be only one solution to this problem, *i.e.* to confine the hypoxia to one part of the lungs, and to administer oxygen to the remaining part in sufficient concentration either to reduce or fully prevent general anoxæmia. If changes in the oxygen saturation of the arterial blood are produced by relatively low oxygen content gas mixtures for the purpose of indicating a relative shift in blood-flow through the hypoxic and the other part of the lungs, then it is inevitable that some general anoxæmia will be present. When, however, the relative shift in blood-flow is determined by measuring changes in the oxygen tension of the arterial blood, the use of higher gas mixtures becomes feasible and general anoxæmia may be completely eliminated.

In the experiments related in the present paper both methods have been used. The results of the "saturation method," in which it was impossible fully to prevent anoxæmia, have been supplemented by those of the "tension method," which is free from this source of error but which requires the development of a polarographic method for measuring the oxygen tension in small samples of arterial blood.

## METHODS

The experiments have been performed on rabbits mostly under urethane anæsthesia (1–1.5 g/kg subcut.), but sometimes pernocton has been used (40 mg/kg intraven.). During the prolonged experiments the anæsthesia had to be supplemented either by subcutaneous and intramuscular injections or by intraperitoneal infusion.

Various methods have been described in the literature for the separate ventilation of sections of the lungs [Heemstra, 1948]. Of these the trachea divider of Dirken and van Dishoeck [1937] has been chosen to effect separate ventilation of the right and the left lung of the rabbit without danger from obstruction of the narrow air-passages.

In the present experiments the lungs were breathing gas mixtures of known and constant composition during periods lasting up to 24 hours and more. These mixtures have been prepared at the rate of about 1.8 litre per minute by allowing the component gases to escape from the storage flasks at a constant pressure through a constant resistance. Commercial reducing valves were used provided with narrow glass or

affords a valuable complement to the saturation method, the more so as general hypoxæmia is fully prevented, and therefore any resulting phenomena are not to be ascribed to this cause

For this purpose the polarographic method [Heyrovski, 1941, Kolthoff and Lingane, 1946] has been extended to the determination of the oxygen tension of the blood. The difficulty encountered in working on blood springs from the presence of dissociable oxygen in the form of oxyhæmoglobin, which prevents the diffusion current from increasing proportionally to the oxygen tension as is the case in plasma. At a given oxygen tension the diffusion current in blood surpasses that in plasma for the same reason. To avoid this difficulty the blood corpuscles were eliminated by centrifuging the blood samples, and the oxygen tension of the hæmoglobin-free plasma was measured. This procedure has also been adopted by Berggren [1942]. In doing so, special care must be taken not to disturb the equilibrium between the dissolved oxygen and that combined to the hæmoglobin, as the amount of the former determines the tension of the oxygen. As this equilibrium is known to be sensitive to changes in temperature, it is obvious to take precautions for keeping the sample at body temperature. However, a new source of error is then introduced, as at this temperature the oxygen consumption of the blood and the plasma is not negligible. This induced Berggren to restrict his experiments to those oxygen tensions which guarantee full saturation of the hæmoglobin at any temperature. This precaution allows him to centrifuge, etc., at  $0^{\circ}\text{C}$  without disturbing the equilibrium of the oxygen in the blood and in this way to exclude any effect of oxygen consumption. Berggren assumes an oxygen tension of 150 mm Hg to be sufficient to ensure full saturation. The shape of the dissociation curve at the higher levels is not very well known, but the results of Brinkman and Dirken [1940] indicate that even at 200 mm Hg there might still exist an appreciable degree of subsaturation. Moreover, the procedure adopted by Berggren eliminates the measurement of oxygen tensions under 150 mm Hg, which in the present experiments would have greatly restricted the applicability of the polarographic method.

For these reasons the attempt was made to maintain the equilibrium between dissolved and combined oxygen by keeping the blood sample at body temperature and reducing the time interval between the sampling and the photographic registration as much as possible. A strict time schedule was kept in order to improve the constancy of the error introduced by the oxygen consumption of the blood and the plasma. As exactly the same procedure was followed in the construction of the calibration curves by which the excursions of the galvanometer were to be converted in mm oxygen tension, the error was still further reduced. The said calibration curves were obtained by polarographic measurement of samples of rabbit's blood equilibrated tonometrically

The accuracy with which a change in the mixing-ratio of the blood from both lungs may be derived from a rise or fall of the oxygen saturation of the mixed arterial blood depends on the difference in oxygen saturation of the blood leaving the lungs. When the blood coming from the oxygen lung is fully saturated, that leaving the nitrogen lung has to drop to 75 per cent saturation, corresponding with an oxygen content of the inspiratory air below 5 per cent, in order to produce a fall of the saturation of the mixed arterial blood to between 85 and 90 per cent. On ventilating the nitrogen lung with mixtures containing from 5 to 10 per cent oxygen, however, the resulting fall of the saturation may only amount to a few per cent. As a subsequent change in

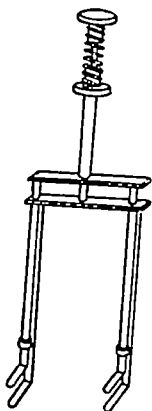


FIG 1—Double clamp for carotid artery



FIG 2—Serrefine, closing the puncture in the wall of the artery

the blood-flow ratio is to be detected by the extent of the return of the saturation to its original level, it is obvious that the saturation method may only become sufficiently accurate when an appreciable degree of subsaturation and of general hypoxæmia is permitted to develop. It was for the purpose of studying the effect of slight grades of hypoxia occurring in one lung that another method was developed on the same lines, in which not the saturation but the oxygen tension of the mixed arterial blood was used as an index for the relative perfusion of the lungs.

*"Tension Method"*—If both lungs are breathing 65 per cent oxygen, the resulting oxygen tension of the carotid blood may be found to attain something like 375 mm Hg. Administering a mixture containing 17 per cent oxygen to one lung may result in a fall of the mixed arterial blood oxygen tension to about 120 mm Hg. Any decrease in the circulation of the "nitrogen lung" as compared with that of the "oxygen lung" will result in a rise of the oxygen tension of the mixed arterial blood from 120 mm Hg to considerably higher values. This example may suffice to demonstrate the tension method to be particularly adapted for studying the effect of slight grades of hypoxia. Thus this method

diameter of the capillary tube is 1 mm, its length 65 cm, and the rubber connexions have been reduced to a minimum. The tip of the glass capillary is drawn out to obtain a drop-time of the mercury electrode of approximately 5 seconds. During the whole of the experimental period, which may last 24 hours or more, the flow of mercury was not interrupted, but on account of the relatively large size of the bulb the fall of the mercury level nevertheless amounted to no more than 2 mm. By the repeated contact with plasma a deposit is formed on the fine tip of the mercury electrode, causing the drop-time to become irregular. This may be prevented by keeping the tip under water between measurements and by submerging it at regular intervals in concentrated nitric acid without, however, interrupting the flow of the mercury. After thoroughly rinsing with water the effect of the cure may be controlled by measuring the drop-time.

The dropping mercury electrode is maintained at a constant potential of  $-0.7$  V, a N-calomel electrode serving as an anode. At this potential the strength of the current is proportional to the oxygen tension of the plasma. The excursions of a micro-mol galvanometer with a period of 0.2 sec and sensitivity regulated by an Ayrton shunt were photographically recorded together with a time signal of 0.1 sec. The records show the variation of the current during each drop-cycle. The strength of the current is read at exactly five seconds from the abrupt decrease in current indicating the fall of the former drop. Thus variations in drop-time are cancelled out, and even slight irregularities in growth of the mercury-drop affecting the reliability of the readings are easily detected. The oxygen tension of the sample may be read from a calibration curve obtained in the same way with known oxygen tensions.

In withdrawing blood from the carotid artery the same procedure was followed as described above for the saturation method. The dead space of the syringe was filled with 0.06 c.c. of a saturated solution of sodium oxalate in equilibrium with atmospheric air. For mixing the blood with the oxalate a small metal ball was enclosed, flattened in places in order to prevent any obstruction in emptying the syringe, which may cause hæmolysis of the blood by forcing it under pressure through the narrow slits left between the surface of the ball and the conical outlet of the syringe.

The blood is transferred to a small thick-walled centrifuge tube contained in a case of palm-wood (fig 3, f). The tip of the needle is placed on the bottom of the vessel and the syringe quickly emptied in order to minimise the effect of contact with air. By this simple expedient the filling of the tubes over mercury as advocated by Berggren is made quite superfluous. When the tube has been filled the cap is screwed on top preventing any further contact between air and blood. The tube in its case is then placed in a Zernicke centrifuge capable of doing 20,000 revolutions per minute.

at 38° C at given oxygen tensions (Brinkman and Dirken, 1940) Moreover, absolute values were not required in the present experiments, as information on changes in blood-flow of both lungs may be derived

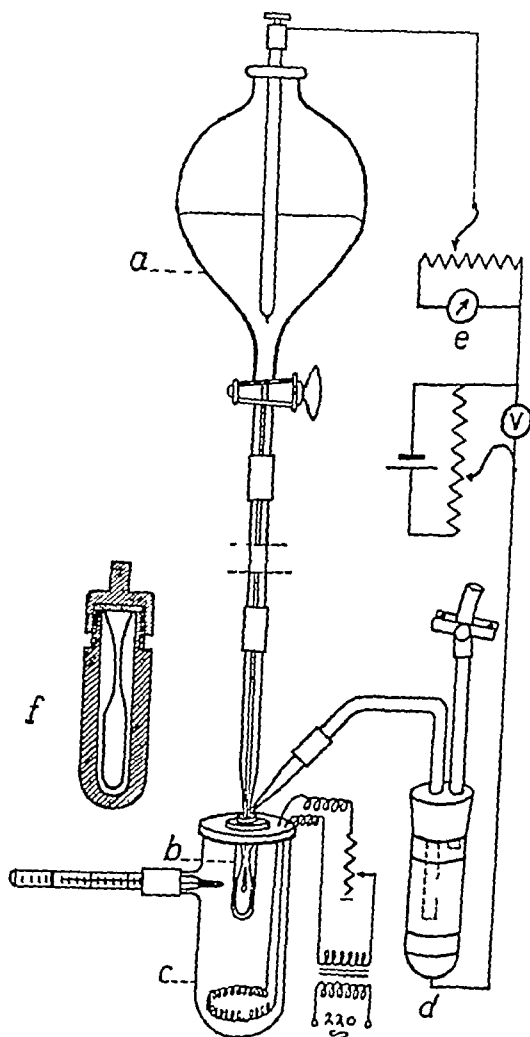


FIG 3 —Polarograph and electric circuit (a) Mercury bulb (b) dropping electrode and sampling tube (c) water bath and heating circuit (d) calomel electrode (e) galvanometer and Ayrton shunt (f) sampling tube in case of palm wood V=voltmeter

from comparison of the oxygen tension of the various blood samples Therefore it sufficed to subject them to exactly the same treatment and to minimise the accidental error

The polarograph is represented schematically in fig 3 The internal

rise of the oxygen tension of the blood from the carotid artery above the original value when, after starting with one lung on atmospheric

TABLE I

Time	Insp air O <sub>2</sub> per cent		Alveolar air (L )		Art sat HbO <sub>2</sub> per cent
			CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
10 40	L 99	R 99			
10 45	L 0 22	R 99			99 8
10 55	"	"	3 14	2 34	
10 58	"	"			90 0
11 10	"	"	3 06		
11 13	"	"			87 5
11 42	"	"	2 98	2 46	
11 45	"	"			89 9
12 40	"	"	3 20	2 08	
12 42	"	"			91 8
13 40	"	"	2 82	2 21	
13 45	"	"			94 3
15 10	"	"	2 73	1 92	
15 15	"	"			95 0
16 40	"	"	2 84		
16 45	"	"			96 4
18 10	"	"	2 60	2 00	
18 15	"	"			99 0
19 43	"	"	2 47	2 08	
19 45	"	"			99 0

TABLE II

Time	Insp air O <sub>2</sub> per cent		Alveolar air (L )		Art sat HbO <sub>2</sub> per cent
			CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
9 45	L 99	R 99			
10 40					100 8
11 00	L " 4 65	R " 99			
11 05	"	"	4 24	4 60	94 6
11 50	"	"	4 09	4 62	94 0
12 50	"	"	3 56	3 94	94 1
14 20	"	"	3 10	4 36	96 2
15 55	"	"	3 14	5 12	97 1
17 20	"	"	3 05	4 75	98 2
18 52	"	"	3 53	4 25	99 2
20 20	"	"	3 48	4 26	100 0
21 50	"	"	3 43	4 64	100 4

air and the other on oxygen, the air was replaced by a gas mixture containing less oxygen

The results of these experiments clearly show that the oxygen

In controlling the temperature of the blood sample stress must be laid on its temperature being  $38^{\circ}\text{C}$  during the period of the separation of the blood corpuscles from the plasma, *i e* during the centrifugation. Although cooling of the sample during the arterial puncture was reduced by the use of a syringe provided with a hot-water jacket, the centrifuge and the wooden case had to be pre-warmed to  $42^{\circ}\text{C}$ . In that case, as was shown in trial experiments by means of thermocouples, the temperature of the sample rapidly regains almost  $38^{\circ}\text{C}$  and after that only rises slowly ( $0.2^{\circ}\text{C}$  per 30 sec). By adhering to a strict time schedule the centrifugation was always accomplished during this period.

Thirty seconds suffice to separate the plasma from the blood corpuscles, and in about one minute the centrifuge is brought to a stand still. The glass tube is now transferred from its wooden case to the small water-bath under the mercury electrode. The capillary is dipped into the wider part of the tube and obstructs for the greater part the narrow neck, thereby minimising the danger of contamination of the contents with air. The ceramic point of the calomel electrode is brought into contact with the surface of the plasma. The whole procedure from sampling to recording is completed within 5 minutes, each step being controlled by chronometer. With these precautions the accidental error of the method proved to be 3 mm Hg [Heemstra, 1948].

## RESULTS

At the onset of the administration of nitrogen to one of the lungs of a rabbit, a sudden drop of the oxygen saturation of the blood taken from the carotid artery is observed. If, then, the composition of the inspired gases, *e g* nitrogen to the left and oxygen to the right lung, is kept constant, this fall is found to disappear spontaneously in the course of some hours. At the end the saturation usually attains a level quite near to that of the beginning of the experiment, when both lungs were breathing oxygen (Table I).

A less severe degree of hypoxia in one lung, such as that produced by the administration of  $\pm 65$  per cent oxygen, also starts the same compensating reaction (Table II).

In these latter experiments, which only involve a slight subsaturation of the blood, the limit of the accuracy of the saturation method is approached. Comparable results of the inhalation of 5 per cent oxygen by one lung have also been observed by means of the tension method (Table III).

Even the administration of gas mixtures containing 15 per cent or more oxygen, causing only a very slight fall of the alveolar oxygen content, is still found to produce a pronounced effect (Table IV).

Often the paradoxical phenomenon has been observed of an ultimate



successively to gas mixtures of different composition Table V and fig 4 show the results of one of these experiments

The oxygen tension of the arterial blood is seen to recover slowly from a drop of the alveolar oxygen tension to 9.17 per cent When this is again lowered to 7.59 per cent, the initial fall in arterial oxygen

TABLE V

Time	Insp air O <sub>2</sub> per cent	Alveolar air				Art pO <sub>2</sub> , mm Hg
		L		R		
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
13 56	L 65 R 65					359
14 10	" "			6 31	61 57	
14 15	" "					371
14 30	" "					373
14 42	L 65 R 14					
14 45	" "					103
15 45	" "					104
16 00	" "	5 52	59 58	5 20	9 17	
18 00	" "					148
19 00	" "					150
20 00	" "					150
21 00	" "					168
22 00	" "					172
23 00	" "					185
24 00	" "					183
0 50	" "					205
2 00	" "					209
3 00	" "					211
4 00	" "					211
4 17	L 65 R 11 3					
4 20	" "			3 15	7 59	155
4 30	" "					
5 20	" "					156
8 00	" "					166
9 10	" "					211
10 45	" "					228
12 00	" "					249
14 15	" "					282
15 00	" "					278

tension is followed by a renewed increase Therefore a relation must exist between the strength of the effect and the extent of the deviation from the normal alveolar tension

In every one of these experiments the oxygen lung was breathing a gas mixture rich in oxygen The question therefore arises whether this ventilation of the oxygen lung with an abnormal gas may not have caused the observed gradual increase of the saturation and oxygen tension of the arterial blood This has been tested in a series of experi-

content of the mixed arterial blood spontaneously recovers in the course of some hours from any fall caused by a lowering of the alveolar oxygen tension in one lung We have tried to establish a relation between the

TABLE III

Time	Insp air O <sub>2</sub> per cent	Art pO <sub>2</sub> , mm Hg
11 30	L 99 R 21	
12 50	" "	294
15 15	L "99 R 4 75	
16 30	" "	76
18 00	" "	113
19 30	" "	128
21 00	" "	155
22 30	" "	250
0 05	" "	292

TABLE IV

Time	Insp air O <sub>2</sub> per cent	Alveolar air (R )		Art pO <sub>2</sub> , mm Hg
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
11 20	L 99 R 22 8			
12 30	" "			247
12 45	" "	4 40	19 33	
13 10	" "			260
13 57	L "99 R 15			
13 59	" "			162
14 10	" "			161
14 30	" "			180
15 00	" "			200
15 10	" "			
16 00	" "	4 90	10 69	243
17 00	" "			256
18 00	" "			310
19 00	" "			303
20 15	" "			325
21 15	" "			330
22 15	" "	3 18	11 94	343
23 30	" "			360
0 30	" "			
0 35	" "			355
1 30	" "			339

size of the fall in alveolar oxygen tension and the rate or the magnitude of the recovery process The individual differences, however, proved to be rather great Therefore a couple of experiments have been performed in which one lung of the same animal has been exposed

## DISCUSSION

The spontaneous recovery of the saturation and of the oxygen tension of the mixed arterial blood from the effect of local hypoxia in one lung may easily be explained by a change in the blood-flow ratio of the lungs resulting in the larger amount of blood being directed to the oxygen lung. Even so, the supposition of a modified diffusion or even of a secretion of oxygen cannot be passed without comment. The possibility too of the special conditions in the nitrogen lung influencing the transport of oxygen by the blood will have to be shortly discussed.

One may suppose the diffusion of oxygen through the alveolar membrane in the nitrogen lung either to be decreased or increased during the recovery process. A progressive inhibition of the diffusion process might improve the saturation and the tension of the arterial blood at very low alveolar oxygen tensions by diminishing the loss of oxygen from the blood. But an improvement is also seen in those experiments in which oxygen is taken up instead of lost by the blood, as is shown by the difference between the alveolar and the inspiratory oxygen tension (Table IV). Moreover, an increase in saturation of the arterial blood up to 99 per cent (Table I) can never be explained in this way. On the other hand, a facilitation of the diffusion of oxygen in the nitrogen lung will never explain the increase in oxygen content of the mixed arterial blood in those cases in which oxygen is lost by the blood on its passage through the nitrogen lung, as is shown in Table I, in which the alveolar oxygen tension is about 2 per cent higher than that of the inspiratory air. An increased loss of oxygen from the blood would be the result. From this one may conclude that the observed phenomenon cannot be explained by an increase or decrease of the diffusion process in the nitrogen lung.

The theory of an active secretion of oxygen occurring in the lungs, first formulated by Bohr and in 1935 in modified form still defended by Haldane and Priestley [1935], has never been generally accepted since Krogh [1910] and Barcroft [1925] investigated the problem. The results of the present experiments too do not support the theory. On breathing gas mixtures poor in oxygen the oxygen content of the alveolar air markedly exceeds that of the inspired gas (Table I), proving that even in these favourable circumstances oxygen is not secreted but is actually being lost by the blood. On the inhalation of 4.65 per cent oxygen (Table II) the content of this gas in the alveolar air equals that of the inspired gas during the whole of the experiment. Notwithstanding oxygen is neither lost nor taken up in the nitrogen lung, and the saturation of the arterial blood is slowly restored to its original level. The experiments involving higher oxygen mixtures, e.g. 15 per cent, too do not support the secretion theory. If during the experiment the composition of the inspired gas is maintained constant, one might expect an increasing

ments in which for hours one lung was breathing atmospheric air and the other pure oxygen

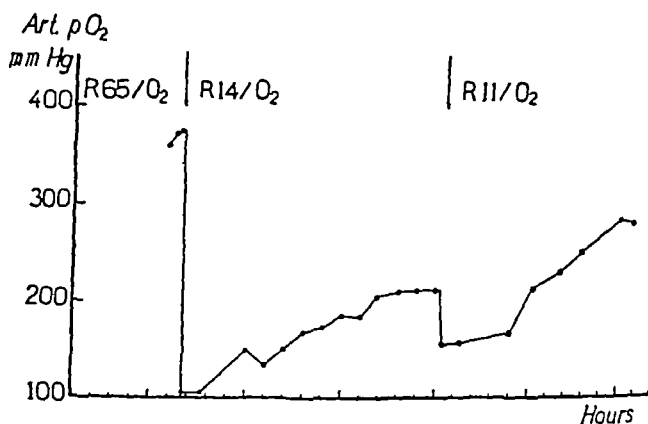


FIG 4—Graph of arterial oxygen tension during unilateral inhalation of 14 and 11 per cent O<sub>2</sub> (Table V)

TABLE VI

Time	Insp air O <sub>2</sub> per cent	Alveolar air				Art pO <sub>2</sub> , mm Hg
		L		R		
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
13 15	L 20 35 R 98					
13 23	" "					315
14 00	" "					328
14 12	" "	4 96	15 74	5 62	91 49	
15 00	" "					334
16 00	" "					317
17 00	" "					357
18 00	" "					347
20 00	" "					358
21 00	" "					345
21 12	" "	4 77	16 14	5 30	91 74	
22 00	" "					343
23 00	" "					330
1 00	" "					316
2 00	" "					355
3 00	" "					300
4 15	" "					328

In the experiment, reproduced in Table VI, oxygen has been administered to the right lung for 15 hours at a stretch without occurrence of a significant change in the arterial oxygen tension. It is obvious that the well-known noxious effect of prolonged inhalation of pure oxygen is negligible under the chosen experimental conditions.

## DISCUSSION

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secretion of oxygen to lower the alveolar oxygen tension of the nitrogen lung. The reverse actually proves to be the case (Table IV). Thus the rise of the arterial saturation in unilateral breathing of 15 per cent oxygen cannot be ascribed to an increasing secretion of oxygen.

All the same, a decrease of the alveolar carbon dioxide tension is shown to develop in the nitrogen lung in the course of the experiment (Tables I, II, IV), and this factor is bound to promote the uptake of oxygen by shifting the dissociation curve to the left. At a constant ventilation the low carbon dioxide tension would thus tend to lower the alveolar oxygen tension. Its effect, however, can only be of minor importance, as in Tables II and IV a decreasing alveolar carbon dioxide percentage is accompanied by a constant or even by a rising alveolar oxygen tension. In the experiments reproduced in Table I the blood is losing oxygen to the alveolar air and the alveolar oxygen tension tends to fall during the experiment. It would appear that this drift in the direction of the inspiratory oxygen tension and the decrease of the alveolar carbon dioxide tension may have a common origin: a reduction of the gas exchange in relation to the ventilation caused by the decrease in blood-flow through the nitrogen lung.

Although in some experiments the so-called oxygen lung was breathing atmospheric air, in others abnormal gases, e.g. pure oxygen, have been administered. In both cases the saturation and the oxygen tension of the mixed arterial blood has been found to recover from unilateral hypoxia. Prolonged administration of pure oxygen itself does not produce the effect, as has been shown by experiments in which pure oxygen has been given to one lung and atmospheric air to the other (Table VI). Thus the primary cause of the effect is not situated in the oxygen lung and has to be looked for in the nitrogen lung.

The obvious solution is that the subnormal alveolar oxygen tension produces an increase of the vascular resistance in the nitrogen lung and thereby directs a greater part of the circulating blood to the oxygen lung. As a result the saturation and the oxygen tension of the mixed arterial blood rises and may attain or even surpass the original level (Table IV).

This conclusion is supported by the results of the analyses of the alveolar air. A reduction of the blood-flow in the nitrogen lung will cause a decrease in the uptake or in the loss of oxygen. In consequence the composition of the alveolar air will tend to shift towards that of the inspiratory air. Indeed, if the inspiratory oxygen tension exceeds that of the alveolar air, a rise of the alveolar oxygen tension is observed (Table IV). If the tension of the inspired gas equals that of the alveolar air, the alveolar oxygen tension remains fairly constant (Table II). Finally, if a loss of oxygen from the blood causes the alveolar tension to exceed that of the inspiratory air, the alveolar oxygen tension in the nitrogen lung is seen slowly to decrease.

The results of the determinations of the alveolar carbon dioxide

concentration again strengthen this conclusion. Of three comparable experiments (one of them reproduced in Table VII) in which the right lung breathed a mixture of 5 l per cent oxygen and the left lung 65 per cent oxygen, the mean alveolar carbon dioxide percentage at the beginning of the experiment amounted to 3.01 per cent (R) and 3.71 per cent (L). The difference between both lungs (0.70 per cent) may be ascribed to the poor oxygenation of the blood in the nitrogen lung, which also impairs the elimination of the carbon dioxide. After more than ten hours of unilateral breathing of 5 l per cent oxygen the mean carbon dioxide percentages amounted to 2.84 per cent (R,) and 4.40 per cent (L). The increase of the difference from 0.70 to 1.56 per cent also points to a reduction of the circulation in the nitrogen lung in comparison with that of the oxygen lung. From this may also be inferred that the ventilation of the nitrogen lung is not diminished to the same extent as its circulation.

The results of the determinations of the alveolar gases may be summarised in saying that all alveolar gas percentages in the nitrogen lung show a tendency to shift towards those of the inspiratory air. This indicates a change in the ventilation blood-flow ratio developing in the nitrogen lung either by an increase in ventilation or a decrease in blood-flow. The ventilation of the nitrogen lung, however, has never been found to show a gradual increase. In many experiments the frequency and the depth of the respiratory movements of each lung have been repeatedly recorded spirographically for short periods of a couple of minutes each. One of these experiments has been reproduced in Table VII, in which a slight decrease of the minute-volume of the ventilation is seen to accompany the recovery of the saturation of the blood. In most cases no distinct change was noted.

In this experiment, as in some others, the composition of the alveolar gases has been measured both in the nitrogen and in the oxygen lung. The alveolar carbon dioxide percentage of the oxygen lung is seen to rise as that of the nitrogen lung diminishes. The difference between the carbon dioxide percentages in the left and in the right lung shows a gradual increase as the arterial saturation slowly regains its original level. All this is in accordance with the supposed increase in blood-flow through the oxygen lung at the expense of that of the nitrogen lung. The alveolar oxygen tension of the nitrogen lung is only slightly lower than that of the inspiratory air, which indicates that the oxygen uptake of this lung is negligible. Therefore during the whole of the experimental period almost all oxygen has to be taken up in the oxygen lung independently from changes in the circulation ratio of both lungs. For this reason no important changes in the alveolar oxygen tensions are to be expected as long as the ratio ventilation/ $O_2$  consumption remains constant.

The phenomenon is not only characterised by its gradual develop-

ment, but also by a slow disappearance when the normal alveolar oxygen tension is restored

In Table VIII a specimen is given of some experiments in which, after the development of vasoconstriction in the nitrogen lung, demonstrated in this case by a rise of the arterial tension from 74 to 142 mm Hg, the initial conditions were restored by switching the right lung back

TABLE VII

TABLE VII

Time	Insp air O <sub>2</sub> per cent	Alveolar air				Tidal air		Freq	Art Sat. HbO <sub>2</sub> per cent		
		L		R		Total cc	Per cent R				
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent						
23 16	L 65 R 65										
23 40	" "					15 3	58 8	30			
23 57	" "					15 2	57 9	31	99 6		
0 17	" "					13 4	58 9				
0 20	" "	4 98	59 46	4 93	60 76						
0 28	" "										
0 31	L 65 R 5 10					13 3	58 6	30			
0 38	" "					13 0	61 5	36			
0 45	" "					12 8	55 5	36	86 8		
2 35	" "										
2 40	" "										
2 50	" "										
4 35	" "	3 82	58 72	3 54	4 91	17 6	56 3	38	90 6		
5 30	" "					13 8	53 6	38			
5 40	" "										
5 50	" "										
7 38	" "	3 99	59 77	2 65	4 82	9 6	51 0	50	90 8		
7 45	" "										
9 35	" "					14 1	56 7	28	95 4		
9 40	" "										
9 50	" "										
11 32	" "	4 62	59 53	3 12	4 53	14 9	57 0	28	95 0		
11 40	" "										
11 50	" "										
13 05	" "	4 50	59 68	2 48	4 62	13 9	54 7	26	97 7		
13 10	" "										
13 20	" "					14 9	56 4	22	98 7		

to atmosphere

to atmospheric air After a preliminary rise of the tension to 223 mm Hg, caused by the still existing predominance of the blood-supply to the oxygen lung, a slow decrease is seen to develop to the level observed at the start of the experiment The disappearance of the vasoconstriction too is a slow reaction taking 4 to 5 hours

Not only the reversibility of the reaction, but also the fact that oxygen tensions slightly below normal affect the blood-flow through the lung (e g 12 per cent, Table IV), lead to the conclusion that the observed



vasoconstriction is not due to a noxious effect of the prolonged local hypoxia. Even in normal conditions an alveolar oxygen tension of 80 mm Hg may prevail in subventilated parts of the lungs, and in that case a restriction of the circulation in these sections is obviously important. In this connexion special stress should be laid on the functional significance of the phenomenon. No effects of injury to the lung

TABLE VIII

Time	Insp air O <sub>2</sub> per cent	Alveolar air				Art pO <sub>2</sub> , mm Hg
		L		R		
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent	
12 15	L 62 R 21					
13 15	" "					173
13 35	" "					168
13 55	" "	4 60	57 28	4 73	16 11	
14 05	L 62 R 10 7					
14 10	" "					74
15 55	" "			3 90	8 98	
2 00	" "					142
2 10	L 62 R 21					
2 14	" "					223
2 26	" "					224
2 46	" "					199
3 15	" "					206
4 15	" "					181
5 15	" "					178
7 15	" "					169

tissues (e.g. œdema) can explain the observed facts, nor could any trace of morphological changes be detected in histological slides. A distinct paleness of the nitrogen lung was the only symptom to be observed after the opening of the thorax at the end of the experiment.

## SUMMARY

By means of a trachea divider the left and the right lung of the rabbit may be made to breathe gases of different oxygen content. If one lung is given a gas of low and the other one of high oxygen content, the saturation or the oxygen tension of the mixed arterial blood in the carotid artery depends on the mixing ratio of the blood from both lungs.

On switching one lung over to a gas of low oxygen content, the initial fall in saturation and in oxygen tension of the mixed arterial blood becomes spontaneously compensated to a major degree in about 8 hours.

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		L		R		Total cc	Per cent R				
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	CO <sub>2</sub> per cent	O <sub>2</sub> per cent						
23 16	L 65 R 65										
23 40	" "					15 3	58 8	30			
23 57	" "					15 2	57 9	31	99 6		
0 17	" "					13 4	58 9				
0 20	" "	4 98	59 46	4 93	60 76						
0 26	" "										
0 31	L " 65 R " 5 10					13 3	58 6	30			
0 38	" "										
0 45	" "					13 0	61 5	36			
2 35	" "								86 8		
2 40	" "					12 8	55 5	36			
2 50	" "	3 82	58 72	3 54	4 91				90 6		
4 35	" "										
5 30	" "					17 6	56 3	38			
5 40	" "					13 8	53 6	38			
5 50	" "	3 99	59 77	2 65	4 82				90 8		
7 38	" "										
7 45	" "					9 6	51 0	50			
9 35	" "								95 4		
9 40	" "					14 1	56 7	28			
9 50	" "	4 62	59 53	3 12	4 53				95 0		
11 32	" "					14 9	57 0	28			
11 40	" "										
11 50	" "	4 50	59 68	2 48	4 62				97 7		
13 05	" "										
13 10	" "					13 9	54 7	26			
13 20	" "					14 9	56 4	22	98 7		

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Evidence is produced that this reaction results from a gradual increase in circulatory resistance of the hypoxic lung caused by a local action of the low oxygen tension. This conclusion is supported by the results of the measurement of the alveolar gas percentages in the rabbit.

To exclude an indirect action on the lung circulation caused by the effects of general hypoxæmia the influence of only slightly subnormal alveolar oxygen tensions was studied. Under these conditions the mixed arterial blood remained fully saturated. To this end a polarographic method was devised for measuring the oxygen tension of the blood.

Even at an alveolar oxygen percentage of approximately 12 per cent, which might normally be present in subventilated parts of the lungs, a distinct reaction occurs.

The increased vascular resistance may be abolished in about 4-5 hours by restoring the normal alveolar oxygen tension.

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THE ADAPTATION OF THE LUNG CIRCULATION TO THE  
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INTRODUCTION

In a previous paper [Dirken and Heemstra, 1948] the oxygen saturation of the carotid blood of a rabbit breathing nitrogen with one lung and oxygen with the other was shown to rise spontaneously in the course of some hours to about the same level as when the animal is breathing pure oxygen with both lungs (Table I). This increase could only be ascribed to a diminishing blood-flow in the "nitrogen lung". The almost complete recovery of the oxygen saturation might lead to the supposition that the circulation through the nitrogen lung became nearly completely obstructed. A simple calculation, however, in which the dissolved oxygen is given its due regard, shows that this is not necessarily so.

Rabbit's blood may combine with 13.2 vol per cent of oxygen (*Tabulae Biol.* III, 480), and as the solubility of oxygen in blood is 0.023, the amount of dissolved oxygen will be 1.8 vol per cent at 600 mm Hg, i.e. at the tension polarographically shown to exist in blood on administration of oxygen to both lungs. The oxygen content of the blood leaving the oxygen lung therefore must amount to  $13.2 + 1.8 = 15$  vol per cent. The saturation of the blood leaving the nitrogen lung breathing 0.22 per cent oxygen can only be roughly estimated. Owing to loss of oxygen from the blood the alveolar concentration rises to 2 per cent, but since it is probable that complete equilibrium is not attained between the alveolar air and the blood under the circumstances existing in the nitrogen lung, 40 per cent saturation is a fair estimate. If the small amount of dissolved oxygen is neglected, 40 per cent saturation corresponds to an oxygen content of 5.3 vol per cent. Suppose now the blood-flow through the left lung to be reduced to 40 per cent of its normal value. As the left lung normally receives about 45 per cent of the output of the right heart, its share is now reduced to 18 per cent and that of the right lung has become 82 per cent. Thus the oxygen content of the mixed arterial blood may be calculated to amount to 13.27 vol per cent. Of this about 13 vol per cent will be combined with the



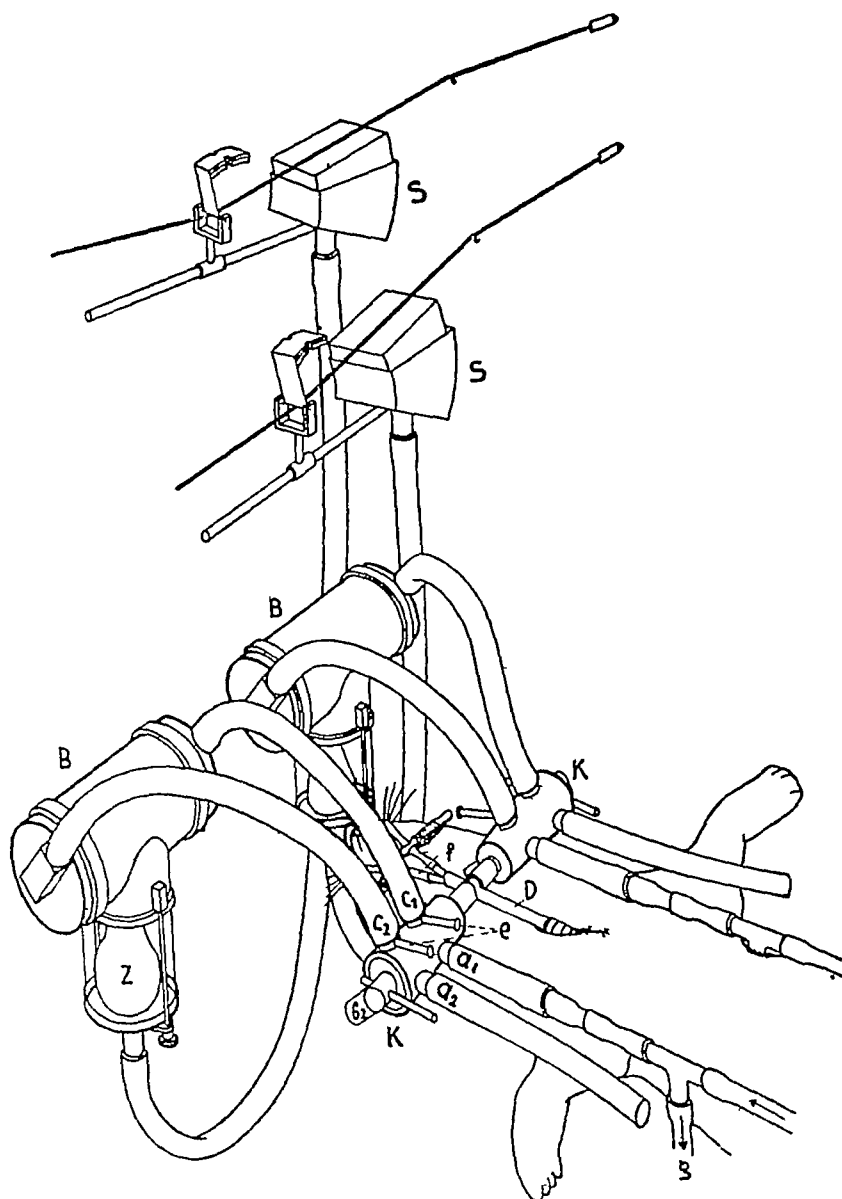


FIG 1—Apparatus for measuring the oxygen uptake of the separate lungs *D* trachea divider *in situ* *B*, absorption vessels *Z*, rubber bag *S* spirometers *K*, membraneless respiration valves for connection with

$a_1, a_2$ —gas mixtures from storage flasks

$b_1, b_2$ —atmospheric air

$c_1, c_2$ —closed systems with  $\text{CO}_2$  absorption

*f* capillary tubes for sampling left and right bifurcation air *g*, to overflow valve

hæmoglobin and so the saturation will be 98 ± per cent. Thus it would appear that the circulation through the nitrogen lung needs only to be reduced to about 40 per cent of its normal value to effect an almost complete recovery of the oxygen saturation of the mixed arterial blood.

The calculation of the circulation ratio of the lungs from data on oxygen tension or saturation, alveolar gas tensions and dissociation curves, can only be approximate. The method was first suggested by Hess [1912] and later developed by Jacobæus and Bruce [1940]. In our opinion the change in arterial oxygen content gives valuable information on the relative shift of the circulation ratio. The absolute error, however, may amount to 20 per cent [Heemstra, 1948]. A much greater accuracy may readily be attained by spirographical methods.

The underlying principle of this method is the proportionality existing between the oxygen uptake and the blood-flow of the lungs, as long as the difference in oxygen content between the arterial and the venous blood and therefore the amount of oxygen taken up by one cc of blood remains constant. For this reason the ratio of the oxygen uptake of the separate lungs may be regarded as an index of the circulation ratio if the oxygen tension in the blood leaving each lung is identical. In the present experiments, however, a different alveolar oxygen tension had to be deliberately maintained in each lung, as the effect of a low alveolar tension in one lung was the subject of the investigation. Therefore the lungs had to be ventilated temporarily with the same gas mixture during the simultaneous measurement of their oxygen uptake. On these lines a "spirographic method" has been developed and a series of experiments have been conducted for the quantitative study of the reaction of the lung vessels to a fall of the alveolar oxygen tension.

## METHODS

The apparatus for measuring the oxygen uptake of the separate lungs has been reproduced in fig. 1. The air passages of the lungs are separated by a trachea divider D and each side is connected to a respiration valve K. The carbon dioxide of the expired air is absorbed by soda-lime in the container B. Previous to each determination the system is flushed with the desired gas mixture through the small tubes *e*. Changes in the volume of the closed system are recorded by a spirometer S via a rubber bag Z. The right and the left lung are connected simultaneously to identical systems. The spirometers are sealed with mercury [Dirken and v. Dishoeck, 1937], smooth movement being assured by covering the mercury with a drop of dibutylphthalate. One of the advantages of a mercury-sealed spirometer is the ease with which the apparatus may be controlled on leaks at any time by putting a small weight on the spirometer bell and thus producing in the system a positive pressure of 5 to 10 cm H<sub>2</sub>O. The valves used are of the membraneless



does not change, the excess ( $4.56 - 1.42 = 3.14$  per cent) must be solely ascribed to the different rate of oxygen uptake of the oxygen and the nitrogen lung, and the difference in oxygen percentage between the inspiratory and the alveolar air (resp. 6.09 per cent and 2.95 per cent) should be proportional to the rate of oxygen uptake. Thus a difference in alveolar oxygen content of 3.14 per cent would result even when both lungs were breathing the same gas. The additional change of the inspiratory air in the course of two minutes' recording is responsible for the increase of the difference in alveolar oxygen content of both lungs to 4-5 per cent.

TABLE I

Oxygen lung	insp. air	17.09% O <sub>2</sub>	alv. air	11.00% O <sub>2</sub>	difference	6.09% O <sub>2</sub>
Nitrogen lung	insp. air	18.51% O <sub>2</sub>	alv. air	15.56% O <sub>2</sub>	difference	2.95% O <sub>2</sub>

In the same manner it may be shown that the alveolar carbon dioxide percentage in the lung with the higher gas exchange may rise to approximately 1 per cent above that of the other lung. This factor too tends to lower the amount of oxygen taken up by each c.c. of blood passing the oxygen lung.

Still another factor will be working in the same direction. The rate of circulation through the more active lung will be increased and so the time available for diffusion will be shortened. Though it has been previously shown [Heemstra, 1948, Dirken and Heemstra, 1948] that the diffusion membrane itself remains unaffected by the experimental conditions, the faster circulation rate combined with the unfavourable shift of the alveolar tensions may well prevent the attainment of full equilibrium.

TABLE II — SHARE OF THE NITROGEN LUNG IN PER CENT OF THE TOTAL OXYGEN UPTAKE

Atmospheric air	52.9	51.0	42.5	42.5	32.2	27.6	27.2
Oxygen	53.3	52.0	37.9	35.1	28.5	22.5	22.0

These various factors coming into action during the recording when the closed systems are filled with atmospheric air, tend to disturb the equality of the oxygen content of the blood leaving both lungs. Owing to the impaired oxygenation of the blood in the lung with the better circulation, the change in the ratio of the oxygen uptake of the lungs will be smaller than the actual change in circulation ratio. Unless special mention is made to the contrary, the results reproduced in the present paper have been obtained with air-filled systems, and therefore the actual blood-flow changes are still more extensive than those reported. By the introduction of a correction factor the true circulation ratio might be derived from the observed values of the ratio of oxygen uptake obtained with air-filled close systems. The true value may be determined by filling both systems with pure oxygen, because a complete saturation of the blood with oxygen will then occur in both lungs. Table II shows the different results obtained with the two procedures.

type [Dirken and Niemeyer, 1935] By incorporating them in a stop-cock a turn of the handle suffices to connect a lung with

- (a) the flow of gas from a storage flask passing the valve via  $a_1$  and  $a_2$ ,
- (b) the atmospheric air through the tubes  $b_1$  and  $b_2$ ,
- (c) the gas in the closed respiratory system by  $c_1$  and  $c_2$

Usually during the experiments one lung is breathing a gas of poor and the other one a gas of high oxygen content For measuring the oxygen uptake they are both connected for a couple of minutes to the closed systems, which have been carefully swept in advance with the same gas At the moment of switching over, however, one lung contains but little and the other much oxygen As a different composition of the gas in both systems would result, atmospheric air is given to both lungs for half a minute before connecting them to the closed system One of the main advantages of the method is the simultaneous registration of the oxygen uptake of the right and the left lung Errors caused by changes in minute-volume of the right heart or by variations in oxygen content of the venous blood are cancelled in this way

The procedure of changing the inspired gas, however, is only permissible if it can be assumed that the circulation ratio of the lungs is not affected in the two or three minutes of recording In a number of cases the rate of recovery from the effects of prolonged local hypoxia in one lung has been investigated A specimen of these experiments has already been reproduced in a former paper [Dirken and Heemstra, 1948, Table VIII] No change in blood-flow through the nitrogen lung has ever been noted within ten minutes of the return to the breathing of atmospheric air Considering that the total recording period takes three minutes at the most, an error arising from this source is not to be expected

As the method requires strict equality of the oxygen content of the blood leaving the right and the left lung during the period of recording, still another source of error may be present, especially when the closed systems have been filled with air instead of pure oxygen In the latter case the blood will undoubtedly become completely saturated in both lungs As, however, in the earlier experiments the closed systems have been flushed with air, the consequences of the different rate of oxygen uptake in both lungs have to be considered If oxygen is taken up from one air-filled closed system at a faster rate than from the other, the oxygen concentrations will differ after some time At the dimensions of the apparatus actually used this difference after full development of the vasoconstriction in the nitrogen lung has been found to amount to 1-2 per cent at the end of two minutes' recording (Table I 18.51 - 17.09 = 1.42 per cent)

The difference in the alveolar oxygen content is, however still greater (Table I 15.56 - 11.00 = 4.56 per cent) than that in the inspiratory air (1.42 per cent) As the ventilation ratio of both lungs

does not change, the excess ( $4.56 - 1.42 = 3.14$  per cent) must be solely ascribed to the different rate of oxygen uptake of the oxygen and the nitrogen lung, and the difference in oxygen percentage between the inspiratory and the alveolar air (resp. 6.09 per cent and 2.95 per cent) should be proportional to the rate of oxygen uptake. Thus a difference in alveolar oxygen content of 3.14 per cent would result even when both lungs were breathing the same gas. The additional change of the inspiratory air in the course of two minutes' recording is responsible for the increase of the difference in alveolar oxygen content of both lungs to 4-5 per cent.

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The values in each vertical column have been determined one shortly after the other, and therefore are representative for an identical state of the circulation in the lungs. The difference between these values gives one an impression of the size of the correction factor. If, for instance, the share of one lung in the total oxygen uptake in breathing air amounts to 27.2 per cent, the blood-flow through this lung may amount to only 22.0 per cent of the total lung circulation. The correction factor apparently shows some variation in individual cases.

### RESULTS

Normally the share of the right lung of the rabbit in the total oxygen uptake amounts to about 55 per cent, and its part in the total ventilation is of the same order (56 per cent). The results spread in individual

TABLE III

Time	Insp air O <sub>2</sub> per cent	Oxygen uptake		Tidal air		Frequency of respiration
		Total c c /min	Per cent R	Total c c	Per cent R	
11 32	L 21 R 21	16.2	54.3	13.5	51.9	39
12 06	" "	16.1	50.9	13.5	51.9	39
12 32	" "	16.4	52.4	13.9	54.7	39
12 34	L 87 R 179					
12 47	" "	16.9	47.9	11.5	53.0	52
13 16	" "	17.0	47.7	10.9	53.2	58
14 17	" "	17.3	42.8	12.6	52.4	56
15 17	" "	16.5	41.8	12.5	52.8	47
16 17	" "	17.3	39.9	13.6	52.2	42
17 18	" "	18.3	39.9	13.9	54.0	43
17 45	" "	18.1	40.3	13.0	52.3	43
18 45	" "	17.2	34.9	14.1	50.4	38
19 45	" "	17.3	33.0	12.2	52.5	50
21 45	" "	15.0	28.7	10.4	51.9	54

cases, but both the oxygen uptake and the ventilation of the right lung hardly ever exceed the limits of 50 and 60 per cent of the total.

An example of the use of the spiographic method in investigating the effect of unilateral hypoxia is given in Table III.

The uptake of oxygen by the nitrogen lung ventilated with 1.79 per cent oxygen is seen to drop in the course of over nine hours from 52 to 28.7 per cent. The ventilation does not change appreciably. A reduction in oxygen uptake of the nitrogen lung to half or even less of its original value is quite a common occurrence. But the true reduction of blood-flow must have been still more extensive, as in this experiment the closed systems had been filled with air. On applying the proper

corrections (cf p 217) the circulation ratio will be found to be approximately 23 per cent. Although in different animals some individual variations have been observed, the final values obtained on prolonged inhalation of gas mixtures with an oxygen content under 5 per cent all lie between approximately 21 and 27 per cent (corrected values).

To the physiologist the reaction of the lung circulation to alveolar oxygen concentrations above 5 per cent must be of greater interest, as the lower tensions will seldom be realised in normal conditions. Two

TABLE IV

Time	Insp air O <sub>2</sub> per cent	R alv air		O <sub>2</sub> uptake		Tidal air		Freq	Art pO <sub>2</sub> , mm Hg
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	Total c c/min	Per cent R	Total c c	Per cent R		
14 37	L 65 R 65			18 1	51 0	14 0	56 4	34	428
14 46	" "								
15 00	" "			18 4	52 7	14 3	57 3	35	
15 10	L 65 R 14								
15 14	" "			18 4	52 2	15 7	58 6	39	116
15 20	" "								
17 17	" "			19 7	49 8	14 3	58 8	42	
17 21	" "								
19 14	" "			17 1	48 0	15 6	58 3	35	182
19 20	" "								
19 30	" "	4 32	10 57						
21 21	" "			18 4	46 2	58 3	16 3	32	
21 39	" "								189
23 15	" "			15 5	47 1	13 8	59 4	37	
23 20	" "								
1 14	" "			15 5	46 4	13 4	59 7	50	
1 19	" "								207
3 14	" "			15 8	44 3	11 9	58 0	50	
3 20	" "								
4 16	" "			15 5	42 6	12 7	56 7	50	
4 20	" "								220

specimens of these experiments, in which the oxygen tension of the blood has also been recorded, have been reproduced in Tables IV and V.

Notwithstanding the relatively slight drop in alveolar oxygen content, a distinct decrease of the oxygen uptake in the nitrogen lung was noted in these experiments. The extent of the fall, however, is much less than that occurring in the experiments in which the oxygen deficiency of the inspired gas was greater. In the main a lowering of the alveolar oxygen content to 10.5 per cent reduces the share of the right lung in the total oxygen uptake from 55 to 40 per cent (corrected 35 per cent). At an alveolar concentration of 13 per cent a reduction from 53 per cent to about 45 per cent (corrected 42 per cent) occurred.

(Table V) In that case the level of the alveolar oxygen tension was presumably no more than 2.5 per cent below normal, and the circulation through the lung reduced by at least 15 per cent of the original value. If, however, atmospheric air is administered to the right lung and oxygen to the left (Table VI) no gradual alteration either in oxygen uptake or in arterial oxygen tension is noticed.

A slight fall in alveolar oxygen tension of no more than 2.5 per cent

TABLE V

Time	Insp air O <sub>2</sub> per cent	R alv air		O <sub>2</sub> uptake		Tidal air		Freq	Art pO <sub>2</sub> , mm Hg
		CO <sub>2</sub> per cent	O <sub>2</sub> per cent	Total c c/min	Per cent R	Total c c	Per cent R		
14 00	L 65 R 65			20.7	50.3	14.8	50.7	33	344
14 11	" "								
14 26	" "			19.7	53.3	13.8	50.7	34	370
14 33	" "								
14 44	L 65 R 17								
14 50	" "			21.6	52.8	14.5	50.4	38	118
14 54	" "								
16 50	" "			20.9	48.4	13.4	49.3	55	162
16 57	" "								
17 30	" "	5.23	12.89					31	
18 47	" "			19.2	47.4	14.9	51.0	31	170
18 54	" "								
20 47	" "			20.6	47.6	15.3	51.0	35	200
20 54	" "								
22 49	" "			22.4	46.9	14.9	50.3	34	209
23 10	" "								
0 48	" "			20.9	47.9	15.1	53.0	34	18
0 54	" "								8
2 50	" "			22.0	44.6	14.2	51.4	40	
2 55	" "								216

therefore suffices to produce in the long run a decrease in blood circulation of the lung, which may amount to 15 per cent. The resulting change in the circulation ratio is also clearly shown by the rise of the arterial oxygen tension.

### DISCUSSION

As the results of the present series of experiments show that the reduction in the lung circulation on breathing a low oxygen mixture may amount to more than half of its normal value, a corresponding change in the colour of the lung was to be expected. In fact, in the majority of cases one may observe a distinct paleness of the surface of the nitrogen lung with regard to that of the oxygen lung by quickly opening the chest of the animal at the end of the experiment. The difference in colour

may also be observed during life by opening the thorax under artificial ventilation of the separate lungs. A picture of this is reproduced in fig 2. On removing the chest wall the difference in colour is seen to diminish in a couple of minutes by the contact with the atmospheric air and even more rapidly by the intensive radiation of the strong light needed for photography.

Not only the difference in blood-content but also the different

TABLE VI

Time	Insp air O <sub>2</sub> per cent	O <sub>2</sub> uptake		Tidal air		Freq	Art pO <sub>2</sub> , mm Hg
		Total c c /min	Per cent R	Total c c	Per cent R		
14 00	L 21 R 21	18.4	51.6	18.6	49.4	35	407
14 30	L " " "						
14 38	L " 60 R " 65						
14 43	L " " "						
14 50	L 21 R 21	16.8	52.4	15.7	46.5	38	
15 00	L " " "						
15 08	L 60 R 21						
15 11	" " "						
15.21	" " "	18.9	51.9	16.0	44.4	40	228
17 19	" " "						
17 25	" " "						
19 17	" " "						
19 25	" " "	20.6	52.9	17.2	46.5	34	226
21 17	" " "						
21 25	" " "						
0 21	" " "						
0 27	" " "	22.3	52.0	19.8	48.7	37	211
3 17	" " "						
3 25	" " "						
6 15	" " "						
6 22	" " "	20.5	51.7	16.7	48.5	39	221
	" " "						
	" " "						
	" " "						

circulation rates in each lung may be visualised in the open-thorax preparation. After the intravenous injection of a saturated solution of Evans' blue the dye is first seen to enter the oxygen lung (fig 3, A, B, C, D). The slower circulation rate combined with the smaller blood content of the nitrogen lung point once more to the conclusion that the circulation in this lung has decreased as compared with that in the oxygen lung.

The paleness of the nitrogen lung indicates a state of contraction either of the arterial or the capillary vascular bed. The point of attack of the low alveolar oxygen tension therefore might be either the capillaries or the smaller arterioles. The larger arterial vessels always contain blood of low oxygen tension, and therefore probably will be less sensitive to changes in the alveolar oxygen tension. Most investigators do not

ascribe contractility to the capillaries of the lungs [Tiemann, 1935, Tiemann and Voigt, 1936, Kilian, Schwörer and Schotzky, 1935, Wagner, 1935, Wearn *et al*, 1934] According to Verloop [1946] the smaller branches of the lung arteries of the rabbit may greatly alter their calibre by contraction of the very well-developed media. The inspection of histological slides prepared by fixation *in vivo* by intra venous injection of formol [Redingius, 1922] was not successful in locating the site of the vasoconstriction. The narrowing of the vascular bed may also have been produced indirectly by contraction of the plain musculature of the lungs [von Gehlen, 1941, von Möllendorff, 1942]



FIG 2 —Open thorax preparation after unilateral nitrogen respiration. Pale nitrogen lung (right lung, left in figure) and darker (reddish pink) oxygen lung

The possibility that the diameter of the smaller lung vessels depended upon the tonus of the plain musculature of the lungs cannot be neglected even if no changes in the ventilation are apparent.

A rapid rise of the blood-pressure in the pulmonary artery on breathing gas of low oxygen content has been reported by von Euler and Liljestrand to occur in animals [1946], and by Motley, Cournand, Werko, Himmelstein and Dresdale [1947] in men. The first named authors suppose the phenomenon to result from a direct action of the low oxygen tension on the vessels of the lungs, and regard this as proving the existence of an adaptation of the circulation to the ventilation of the lungs. A rapid reaction of this kind, appearing within a couple of minutes of exposure to a low oxygen mixture, has not been observed in our experiments. Sudden changes may have passed undetected by both methods previously described [Dirken and Heemstra, 1948]. A vasoconstriction lasting some minutes after the cessation of the hypoxia, however, ought to have been detectable by the method described in the



present paper Also, if the acute vasoconstriction observed by von Euler and Liljestrand is a purely local phenomenon, the initial drop of the arterial saturation to 80 per cent and less at the beginning of unilateral nitrogen breathing ought in our opinion to have been less

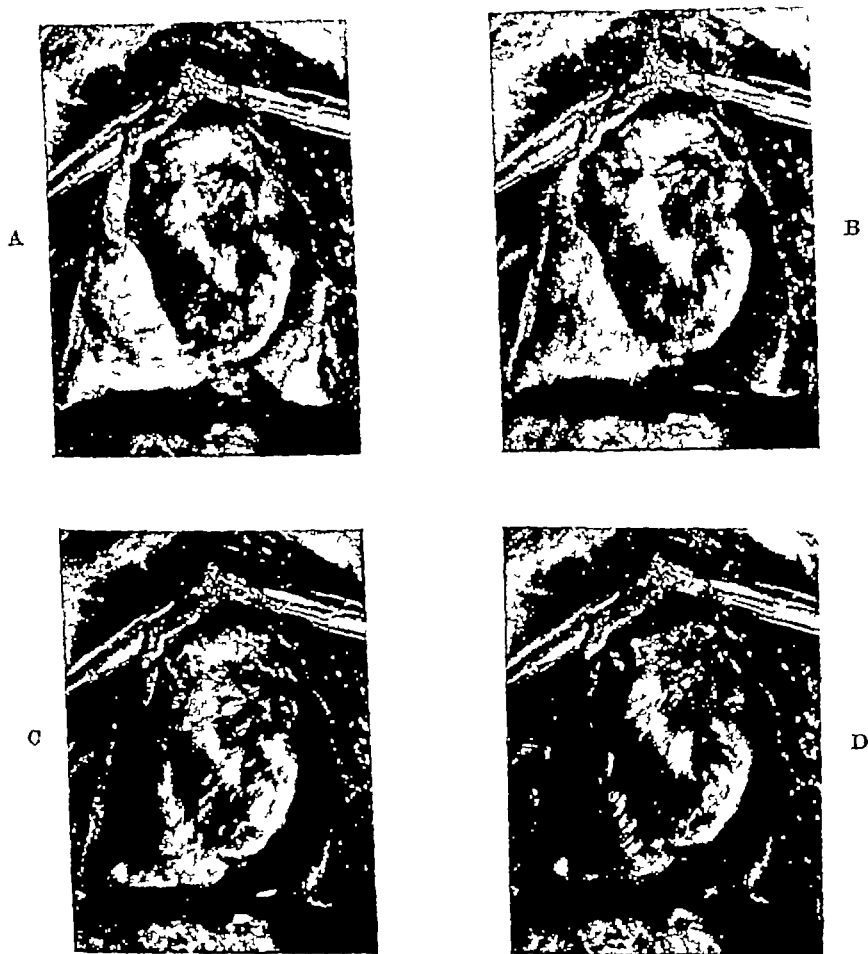


FIG 3 —Penetration of Evans' blue in the oxygen lung (left lung right in figure) and nitrogen lung after intravenous injection

- A. After 6 sec —first coloration in the top of the oxygen lung
- B 8 sec —oxygen lung slightly blue, only a trace of colour in the nitrogen lung
- C 10 sec —oxygen lung deep blue, nitrogen lung slightly blue
- D 12 sec —oxygen lung deep blue, nitrogen lung blue

extensive Moreover, the rise in pulmonary pressure observed by these authors might quite well be ascribed to the effects of anoxæmia in other parts of the body, as no precaution has been taken by them to prevent this complication A change in the circulation *ratio* of the left and right lung as described in the present paper cannot be caused by nervous,

humoral, or hæmodynamic influences resulting from the action of a general hypoxæmia in the systemic circulation. The slow vasoconstriction has been shown to develop in one lung only even when the mixed arterial blood is fully saturated with oxygen, and therefore must be due to a strictly local process occurring in the nitrogen lung.

Both the extent of the reaction and its sensitivity to slight changes in alveolar oxygen tension bring to the fore the problem of the physiological significance of the phenomenon. For some time arguments have been brought forward in favour of the inequality of the ventilation of various sections of the lungs, notably by Haldane and Priestley [1935], Sonne [1936, 1940], Roelsen [1938], and Rauwerda [1946]. An unequal filling of the pulmonary vascular bed too has been observed either by inspection of histological preparations [Cohnheim and Litten, 1875, Toyama, 1925, Kilian, Schwörer and Schotzky, 1935], or by direct microscopy of the living lung [Wearn *et al.*, 1926, 1934, Tiemann and Voigt, 1936, Reinhardt, 1934]. A correlation between the ventilation and the blood circulation in the various parts of the lungs has as a result often been suggested. With regard to the mechanism underlying an adaptation of this kind, mechanical factors have been suggested and their action has been studied in experiments with artificial respiration and perfusion [Tigerstedt, 1903, Wiggers, 1921, de Burgh Daly, 1930]. The decrease in lung circulation resulting from collapse of the lungs and from resorption atelectasis has been demonstrated by Moore [1931], Törning [1933], Dirken, Kraan, Oostinga and Woudstra [1942], and others. In these circumstances mainly mechanical factors are involved.

The results of the present series of experiments, however, do suggest that the ventilation may also influence the circulation in a portion of the lungs by affecting its alveolar oxygen tension. In this way the local circulation may be connected with the local ventilation. If part of the lungs is being subventilated with respect to its circulation, the alveolar oxygen tension will tend to fall. After some time this will result in a decrease of the local blood-flow and the alveolar tension will rise again.

This state of affairs has been expected by Haldane and Priestley [1935, p. 208], suggesting it to be "probable indeed, that in some way or other the air-supply is proportioned to the blood-supply under normal conditions, whether by regulation through the muscular coats of the bronchioles or regulation through the blood distribution, but it is also certain that this adjustment is only an approximation." Indeed, the adaptation obviously is unable to effect complete homogeneity of the alveolar air of the lungs. The inhomogeneity of the alveolar air, resulting from an unequal ventilation, will, however, tend to become less pronounced. In this manner the supply of oxygen to the body is profiting in two ways. In the first place, less blood is supplied to the subventilated sections and is being equilibrated with the low oxygen tension of the alveolar air. Secondly, the alveolar oxygen tension itself is partly restored and there-

with the oxygen saturation of the blood leaving the subventilated sections. The results of the present experiments show that even in cases of extreme inhomogeneity of the alveolar air a subsaturation of the arterial blood may be prevented in this way.

### SUMMARY

In normal circumstances 55 per cent of the total blood supply to the lungs of the rabbit is directed to the right and 45 per cent to the left lung. On lowering the alveolar oxygen tension in one lung its blood circulation may be gradually reduced to less than half of the original value, while the circulation of the other lung shows a corresponding increase.

A drop in the alveolar oxygen percentage of 2 to 2.5 per cent in one lung may reduce the blood-flow by 15 per cent of the original value.

The significance of these results with reference to an adaptation of the circulation to the unequal ventilation of the lungs is discussed.

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AGENTS ACTING ON THE LUNG CIRCULATION By M N J  
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INTRODUCTION

IN previous papers [1948 *a*, 1948 *b*] the authors have described three methods for comparing the circulation in one lung of the living rabbit with that of the other. By means of a trachea divider the animal was made to breathe a different gas mixture with its right and left lung. Under proper conditions either the oxygen saturation or the oxygen tension of the mixed arterial blood, or the uptake of oxygen by the separate lungs, may furnish valuable information on the circulation ratio of the lungs. In addition to permitting the investigations to be performed on the living animal instead of on surviving organs, these methods enable one to discriminate between more general factors affecting both lungs and local factors acting on one lung only. A local vasoconstriction has been shown to develop gradually in one lung on decreasing its alveolar oxygen tension, while the other was breathing oxygen or atmospheric air.

In the present paper the local effects of nervous and humoral agents on the circulation of the lung are described. At the same time a report is given of attempts to obtain an insight in the mechanism of the local reaction of the vessels of the hypoxic lung. In one group of experiments the effect of cutting the vagus nerve and of partial excision of the sympathetic trunk has been studied. In a second series the reaction of the vascular bed has been investigated on local application of various drugs introduced in the lungs as aerosol.

METHODS

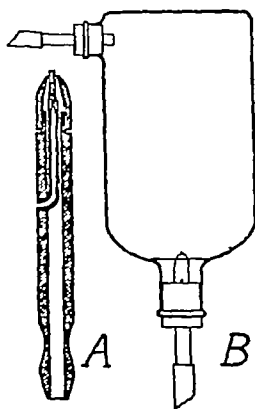
The experiments have been performed on rabbits during urethane anaesthesia (1 to 1.5 g/kg subcut). All three methods previously described—the “saturation method,” the “tension method,” and the “spirographic method”—have been applied indiscriminately, as sometimes the use of the one, sometimes that of the other, proved to be more advantageous.

Various investigators [Larsell, 1921, Dijkstra, 1938, *et al*] have demonstrated the existence of peripheral ganglionic cells in the lungs, and others [Möllgaard, 1912, Dixon and Ransom, 1912, de Burgh Daly and Hebb, 1942] have furnished evidence that part of the nerve-fibres to the lungs are crossing from one side to the other. It would seem, therefore, that it is impossible fully to denervate the lung, and also that the effects of unilateral section of extrapulmonary nerves will not be restricted to one lung. Uni- and bi-lateral vagotomy, uni and bi-lateral extirpation of the sympathetic chain down to T 1 and T 2 (sometimes to T 3 and T 4), both with and without degeneration of the peripheral branches, have been performed in order to study their effects on the lung circulation.

As it was regarded to be of prime importance that the lungs should remain completely uninjured and the respiratory movements not in any way affected by the surgical procedure, a special technique for the resection of the sympathetic has been developed. The cervical sympathetic is dissected and the caudal stump is freed from the surrounding tissue down to the upper aperture of the thorax. At this stage the phrenic nerve is seen running at the upper edge of the area, and special care has to be taken not to injure the nerve by pressure or other interference when trying to enlarge the field of vision. If required, room may be made by removing a section of the clavicle. From now on the continuation of the operation requires some experience, as the very thin sympathetic chain is bending over the vertebral part of the first rib in a dorsal direction. The region of the lower cervical ganglion has been reached, which may be recognised by the presence of a great number of ramifications. After sacrificing the ventral branch of the ansa subclavia the dorsal one is followed down to the first thoracic ganglion, which presents itself as a fusiform thickening of the trunk provided with many branches. At the level of the first rib the vertebral artery is running along the sympathetic chain and has to be cautiously guarded. After severing one by one all connexions of the stellate ganglion, the free end of the nerve is drawn through a small steel ring provided with a cutting edge. The small ring-shaped knife attached to a long, thin handle may be pushed along the nerve following the back wall of the thoracic cavity in the neighbourhood of the vertebral column. By these means it is possible to cut the branches and the rami communicantes of the second and third and sometimes even of the fourth thoracic ganglion without any danger of perforating the parietal sheet of the pleura. As the resection with the aid of the sharp ring has to be performed without visual control, the sympathetic trunk itself is cut finally, usually between the 3rd and the 4th thoracic ganglion. The whole of the operation may be performed without significant loss of blood and without major disturbance to the respiratory mechanism.

In the second series of experiments the local action of various

humoral agents on the lung-vessels has been studied. The substances have been applied to one lung by unilateral inhalation in the form of an "aerosol" [Dautrebande *et al*, 1941]. The apparatus devised for producing these aerosols is reproduced in the figure. Through the central tube of the atomiser (A) the fluid is sucked to the narrow-bore tip and on leaving this is atomised by the rapid gas stream passing around it. To get a stable suspension of very fine droplets the exact position of the air outlet with regard to that of the fluid is highly important. Optimal adjustment may be attained by turning the threaded head of the atomiser and fixing it afterwards by soldering. The atomiser is then mounted in the neck of a bottle (B) in such a way that the inlet of the



Preparation of aerosols A Atomiser B Arrangement.

central tube comes flush with the surface of the stopper. As the jet of atomised solution squirts against the bottom of the flask, the larger particles are caught and run back along the walls. None but the finest droplets are sufficiently slowed down to be able to escape through the side tube. The gas mixture which the animal is breathing passes through the atomiser, thence to the membraneless respiratory valves, so that the aerosol is added to the inspiratory air. The mean size of the fluid particles is estimated to be approximately 0.001 mm. The stability of the mist has been improved by adding 10 per cent glycerol to the solution. This proved to be very satisfactory, as even after hours of passage through a rubber tube of no more than 5 mm internal diameter no inconvenience of any kind has been experienced from an accumulation of fluid. According to Findeisen [1935] a particle size of 0.001 mm is optimum for reaching the ductuli and sacculi alveolares, and over 80 per cent of the aerosol is caught there. The ability of the smaller particles (0.00003 mm) to penetrate would be less on account of their more intensive Brownian motion. Robertson [1941] mentions that the penetration of fine dust and liquid particles to the alveoli has been

ascertained by various investigators. This means there is some justification for the assumption that in the present experiments a considerable part of the aerosol is deposited in the finer ramifications of the air passages. Therefore the various agents may be expected not only to act on the bronchi but also on the smaller blood-vessels and on the parenchymatous tissue of the lung.

The fluid to be atomised must contain a rather great amount of the active substance in comparison with the concentration commonly present in injection fluids. One c c of fluid corresponds with about 25 l aerosol. These 25 l pass the respiratory valves in 15 minutes, and on estimation no more than 2.5 l enter the alveoli ( $\frac{2}{3}$  of the tidal air). Moreover, one-half of the incoming particles may be caught by the bronchial tree. Therefore in starting from a solution containing, say, 5 mg of adrenaline per c c, no more than 16  $\gamma$  per minute may be expected to reach the parenchymatous tissue of the lung.

## RESULTS

*Nervous Influences* —The literature on the action of the vasomotor nerves of the lungs has been reviewed by de Burgh Daly [1933]. The results of the experiments with artificial perfusion show that a vasoconstriction, or less commonly a dilatation, develops on stimulation of the sympathetic. Stimulation of the parasympathetic nerves produces conflicting results. From the more recent work of Daly and co-workers [1942], it is evident that perfusion experiments demand extremely rigid control conditions and a highly specialised technique.

In experiments on the living animal, the resection of the sympathetic chain, including the upper thoracic ganglia, is found to cause an increase in the uptake of oxygen and therefore a rise in blood-flow in the homolateral lung (Table I).

In this experiment part of the right sympathetic, including the first thoracic ganglion, was removed. Between the measurements both lungs were ventilated with atmospheric air, but the records themselves were taken during the inhalation of pure oxygen. The sympathectomy produces a slight but distinct rise in the share taken by the right lung in the total uptake of oxygen. In our experience a reaction of this magnitude usually occurs. In some experiments we have tried to stimulate the upper thoracic ganglion with a faradic current. Although in most cases a diminution of the oxygen uptake of the homolateral lung was effected, this result is not to be regarded as conclusive evidence of the development of a vasoconstriction. Usually the reaction only appeared at a strength of the stimulating current, which by producing ambient currents caused contraction of various muscles and thereby interfered with the normal breathing.



Section of the vagus nerve did not affect the blood-flow through rabbits' lungs. In unilateral vagotomy, followed by removal of the homolateral upper thoracic sympathetic ganglia, the effect of the latter predominated, as the oxygen uptake of the homolateral lung was observed to increase from 53 to 60 per cent of the total value.

In a series of experiments, the effect of dissection of the extrapulmonary nerves on the development of the slow vasoconstriction, caused by a fall in arterial oxygen tension, has been investigated in order to ascertain if the adaptation of the lung circulation to its alveolar  $O_2$  percentage, previously described by the authors [1948 a, 1948 b],

TABLE I \*

Time	Oxygen uptake		Tidal air		Frequency
	Total c c /min	Per cent R	Total c c	Per cent R	
11 55	17 0		14 1		32
12 05	16 4		13 6		32
12 15	15 6	57 7	11 0	58 2	28
12 25	15 0	56 0	11 4	57 0	28
13 20	15 3		13 6		34
14 20-15 00					
Excision of the right sympathetic					
15 10	16 8		13 7		45
15 15	16 9	58 6	11 2	57 1	40
15 50	16 6		13 4		43
16 00	16 2	62 3	10 5	58 1	40
16 40	16 5		13 3		42
16 55	16 4	61 0	10 1	57 4	40

\* Both spirographic systems filled with oxygen

is fundamentally a nervous reaction. The result proved to be negative. Neither uni- or bi-lateral sympathectomy, nor uni- or bi-lateral vagotomy, nor any combination of the two either shortly after the dissection or after a period of 5 to 16 days to allow for time for nerve degeneration, produced the slightest effect on the development of the vasoconstriction caused by the hypoxia of one lung. Therefore the conclusion appears to be justified that extrapulmonary reflexes are not involved in this reaction. Final judgment, however, must be suspended, as according to some investigators [*inter alia* Dijkstra, 1938] nerve-fibres even from the 4th to 7th thoracic ganglia may pass to the hilus of the lung. The negative results of the experiments with nerve degeneration argue against a part being played by axon-reflexes *via* postganglionic sympathetic and preganglionic parasympathetic fibres. Resection and subsequent degeneration is, however, insufficient fully to exclude the interference of axon-reflexes, as numerous intrapulmonary ganglion cells remain unimpaired [Dijkstra, 1938].

*Humoral Influences*—The local effect of humoral agents on the lung-vessels of the living rabbit may be studied by the methods described previously if one succeeds in applying them to one lung only. Gases like carbon dioxide may be added to the inspiratory air and be administered to one lung by means of a trachea divider. The same procedure is practicable with atomised fluids, aerosols. On these lines experiments have been conducted with (a) carbon dioxide, (b) adrenaline, (c) acetylcholine, and (d) histamine.

(a) *Carbon Dioxide*—According to Fleisch and his associates [1932], dilatation results from the direct action of carbon dioxide on the peripheral blood-vessels. A rise of the pulmonary arterial pressure after the inhalation of carbon dioxide has been observed by Binet and Bourlière [1941], and von Euler and Liljestrand [1946]. As this rise may be accompanied by a decrease of the perfusion of the lungs, it is ascribed to a pulmonary vasoconstriction [Diebold, 1937]. Löhr [1924] obtained a vasoconstriction by carbon dioxide in the isolated perfused lungs of cats, but vasodilatation in the presence of traces of adrenaline in the perfusion fluid. In a previous paper [1948 a] the present authors describe a decrease of the alveolar carbon dioxide tension accompanying the vasoconstriction caused by lowering the alveolar oxygen tension. Although it was obvious that the oxygen and not the carbon dioxide tension is the primary cause of the reaction of the vessels, the possibility remained that the change in the alveolar carbon dioxide tension too might affect the blood-flow through the lungs.

This possibility has been tested by the addition of carbon dioxide to the inspiratory air. One of these experiments, in which both the tension and the spiographic methods have been applied, is shown in Table II.

On inhalation of 5 per cent carbon dioxide by the right lung, the arterial oxygen tension is seen to rise from 370 to 403 mm Hg when the lung is breathing 46 per cent oxygen. An increase from 220 to 270 mm Hg is noticed when 21 per cent oxygen is given to the lung. This might be taken to point to a constrictor action of carbon dioxide on the vessels of the right lung, as a rise in arterial oxygen tension will result from a change of the circulation ratio in favour of the left lung, which is inhaling the higher oxygen concentration. This interpretation, however, cannot be accepted without reserve, as a corresponding decrease in the uptake of oxygen by the right lung is not shown by the spiographic method. This discrepancy may be due to a rapid disappearance of a vasoconstrictor action of carbon dioxide, as the spiographic record has to be taken during a period of breathing carbon-dioxide-free gas from the closed systems. This problem can only be solved by a gas analysis method which should provide a continuous record of the oxygen uptake without interruption of the breathing of carbon dioxide. In the meantime, in trying to explain the observed increase in

arterial oxygen tension, one should reckon with the effect of the enlarged ventilation, and also with the more general effects known to result from the breathing of carbon dioxide. One of these is the central and peripheral action of carbon dioxide on the vascular bed in other parts of the body which indirectly may affect the blood-flow through the lungs

TABLE II

Time	Insp air			Alveolar air		Oxygen uptake		Tidal air		Freq	Art pO <sub>2</sub> mm Hg
	O <sub>2</sub> per cent		CO <sub>2</sub> per cent R	CO <sub>2</sub> per cent	O <sub>2</sub> per cent	Total c c / min	Per cent R	Total c c	Per cent R		
	L	R									
14 41	68	46	0			17 1	50 3	15 2	54 0	29	
14 49	"	"	"			17 5	50 9	14 7	53 0		370
14 55	"	"	"								
15 06	"	"	5								
15 12	"	"	"			18 7	51 9	15 6	53 9	39	403
15 16	"	"	"								
15 28	"	21	0								
15 46	"	"	"			18 4	51 6	16 5	53 9	35	222
15 53	"	"	"								
15 54	"	"	5								
16 05	"	"	"			17 5	52 0	16 8	54 1	44	270
16 08	"	"	"								
16 20	"	14	0								
16 25	"	"	"			17 4	49 4	16 8	54 1	34	146
16 29	"	"	"								
16 30	"	"	5								
16 40	"	"	"			16 6	48 8	18 2	53 3	44	151
16 44	"	"	"								
17 11	"	"	"			16 0	45 6	18 0	54 4	68	197
17 16	"	"	"								
17 25	"	"	"	R 5 81	10 61						
17 42	"	"	"			17 9	45 8	19 2	54 7	60	201
17 47	"	"	"								
18 41	"	"	"			16 7	42 0	16 2	53 1	62	232
18 46	"	"	"								
19 41	"	"	"			15 5	43 2	16 0	55 0	55	237
19 45	"	"	"								
19 55	"	"	"	L 3 40	65 71						
21 41	"	"	"			12 9	39 5	14 0	54 3	52	249
21 46	"	"	"								
0 10	"	"	"			9 6	40 6	12 2	54 1	42	264
0 15	"	"	"								

As the available evidence points to the development of a vasoconstriction following a rise of the alveolar CO<sub>2</sub> tension, a decrease of this tension, as has been observed in the afore-mentioned experiments, cannot be the cause of the vasoconstriction developing in a lung whose alveolar oxygen tension has been lowered. This gradual vasoconstriction also occurs in the presence of a high alveolar carbon dioxide tension (Table II), and is seen to pursue its normal course. Therefore the slow

reaction in the hypoxic lung must be fully independent of the alveolar carbon dioxide tension

(b) *Adrenaline*—Usually a constrictor effect of adrenaline on the lung-vessels is reported in the literature. Most of the experiments have been performed with artificially perfused lungs, as in the more recent work of de Burgh Daly and his co-workers [1938, 1940, 1942]

In a series of experiments with the spirographic method, an adrenaline aerosol has been added to the inspiratory air. One c.c. of the atomiser fluid was made to contain 5 mg. adrenaline hydrochloride, corresponding on estimate with an effective dose of 16  $\gamma$  per minute. In Table III an experiment of this kind has been reproduced. The result of the application of the adrenaline-aerosol to the right lung

TABLE III.

Time		Oxygen uptake		Tidal air		Frequency
		Total c.c./min.	Per cent R	Total c.c.	Per cent R	
10 40	R Adrenaline aerosol	16.4	54.3	13.7	57.7	32
11 00		17.2	56.4	13.8	58.0	31
11 02						
11 30		16.2	49.4	12.3	56.1	31
12 00		17.2	44.8	11.5	54.8	34
12 09	R Aerosol discontinued	16.5	46.7	11.5	55.6	34
12 45		16.1	47.8	11.2	53.6	43
12 53		17.1	45.6	11.4	51.8	40
12 55						
13 25		16.2	48.8	12.1	53.7	36
14 25		15.2	53.9	11.3	52.2	35
15 20		16.0	56.3	10.6	53.3	50

is seen to be a diminution of its share in the total oxygen uptake from 55 to about 46 per cent. For reasons given previously, this must be due to a relative decrease in blood-flow. A slight fall in relative ventilation is also observed which, however, is too small to be held responsible for the decrease in blood-flow. As, according to de Burgh Daly, Hebb, and Petrovskaja [1942], a bronchoconstrictor effect of adrenaline in contrast with its common bronchodilator action is a rare occurrence, the slight decrease in ventilation may possibly be ascribed to changes in the elasticity and the viscosity of the lung ("hurdance") [Bayliss and Robertson, 1939]

The effect of ergotamine on the vasoconstrictor action of adrenaline has not been investigated. From the literature, a weakening or even a reversal of the process might have been expected [Daly and Euler, 1932, Foggie, 1937, von Euler, 1932]. On the other hand, we have looked for an effect of ergotamine on the vasoconstriction produced

by the lowering of the alveolar oxygen tension. No effect has been noticed. From this and from the relatively minor reaction on inhalation of the aerosol is concluded that adrenaline is not involved in the production of the vasoconstriction caused by local hypoxia. Moreover, the slow disappearance of this effect (4 to 5 hours) argues strongly against such an hypothesis.

(c) *Acetylcholine* — Various authors mention that acetylcholine exerts a different action with high and with low doses. In general a small dose seems to produce vasodilatation, and a large one constriction, of the vessels of the lung. In artificially perfused lungs of the rabbit a

TABLE IV

Time		Oxygen uptake		Tidal air		Frequency
		Total c c /min	Per cent R	Total c c	Per cent R	
12 00	R Acetylcholine aerosol	12 0	56 7	12 5	54 4	28
12 30		13 2	57 6	13 0	56 9	30
13 00		11 8	54 2	13 3	54 9	30
13 20						
13 40	R Acetylcholine physo stigmme aerosol	12 3	59 3	12 7	55 1	30
13 45						
14 00		13 2	52 3	13 4	53 0	34
14 30	R Acetylcholine physo stigmme atropine aerosol	12 6	36 5	14 0	35 7	36
14 36						
14 41		12 8	39 3	13 4	41 1	38
14 56		13 2	52 3	12 5	49 6	40
15 27	R Aerosol discontinued	12 5	52 8	12 4	50 0	39
15 32						
16 38		12 0	55 8	11 7	52 1	37

strong vasoconstriction appears on the administration of a moderate dose [von Euler, 1932]. In dogs' lungs a dilatation of the vessels is common, but with high doses (over 50  $\gamma$ ) Berry and Daly [1931] have observed a vasoconstriction which was found to increase on giving physostigmine and was abolished by atropine. Foggie [1940] reports identical results. Petrovskaja [1939] mentions that in the lungs of young pigs the dilatation may be converted into constriction by raising the dose or by the addition of physostigmine. A review of the literature on the subject before 1933 has been given by de Burgh Daly [1933].

A specimen of the experiments on the action of acetylcholine on the lung of the living rabbit has been reproduced in Table IV.

The acetylcholine-aerosol (2.5 mg acetylcholine hydrochloride per c c atomiser fluid) without and with the addition of physostigmine (0.3 mg physostigmine sulphate per c c atomiser fluid) is acting on the

right lung during bilateral inhalation of atmospheric air. Without the physostigmine the acetylcholine is seen to produce a weak vasodilatation as the share of the right lung in the total oxygen uptake increases from 56 to 59.3 per cent. The addition of the physostigmine causes a very pronounced vasoconstriction shown by the drop in the oxygen uptake from 59.3 to 36.5 per cent within 45 minutes. The effect is, however, accompanied by an equal decrease of the ventilation of the lung. This result is nearly completely abolished by the addition of atropine to the aerosol (10 mg atropine per c.c. atomiser fluid). In the absence of physostigmine an increase in acetylcholine content of the atomiser fluid up to 10 mg per c.c. does not produce a vasoconstrictor effect (Table V).

TABLE V

Time		Oxygen uptake		Tidal air		Frequency
		Total c.c./min	Per cent R	Total c.c.	Per cent R	
12 00	R. Acetylcholine aerosol	15.1	52.3	14.8	54.1	30
12 28		13.2	51.5	13.4	52.2	30
12 30						
12 40		13.9	52.5	14.0	52.1	30
13 00		13.8	54.3	13.4	53.7	32
14 03	R. Aerosol discontinued	14.1	55.3	14.0	51.4	32
15 00		14.1	58.2	14.0	50.7	30
15 02						
15 30		15.1	56.9	14.4	52.1	35

The gradual development of the dilatation caused by the acetylcholine is rather remarkable. As the hydrolysis of acetylcholine is known to be a rather rapid process, this slow reaction might be an effect of the choline. Experimental evidence, however, showed that choline even in a high dose (150 mg choline per c.c. atomiser fluid) produces no dilatation but only a slight constrictor reaction. On account of the striking result of the addition of physostigmine to the acetylcholine-aerosol the action of this drug has been investigated separately. An aerosol of even 1 mg physostigmine per c.c. atomiser fluid, however, appears to produce only a very weak constriction, decreasing the relative oxygen uptake from 53.5 to 51 per cent.

On the supposition that the vasoconstriction resulting from lowering the alveolar oxygen tension might be due to an accumulation of acetylcholine in the hypoxic lung, the influence of atropine-aerosol on the development of the vascular reaction has been studied. Atropine, which promptly abolishes the striking effect of an acetylcholine-physostigmine-aerosol, proves to be unable to affect the hypoxic vasoconstriction. This reaction of the lung-vessels is neither accelerated nor intensified by physostigmine. The conclusion therefore seems to be

justified that the narrowing of the vascular bed of the hypoxic lung is not to be ascribed to an action of acetylcholine

(d) *Histamine* —The older literature on histamine has been reviewed by Feldberg and Schulf [1930], more recent information on the action of this drug on the vessels of the lungs may be found, amongst others, in the papers of Dixon and Hoyle [1930], Woodbury and Hamilton [1941], Daly [1938], and Daly, Foggie and Hebb [1940] In general the response of the lung-vessels of the rabbit on the administration of histamine turns out to be a vasoconstriction The contraction is supposed to be situated in the arterioles, a specific action on the capillaries is regarded as problematic Our relatively poor knowledge of the action of drugs on rabbits' lungs probably results from the fact that this knowledge had to be derived from experiments with artificial perfusion Rabbits' lungs are not well suited for experiments of this kind

In studying the effect of a histamine-aerosol on the lung circulation of the living animal, a very pronounced diminution has been observed in all experiments

Table VI shows some results obtained by the spiographic method when an aerosol (23 mg histamine dihydrochloride per c c atomiser fluid) was added to the inspiratory air An extensive fall in the circulation of the right lung is noticeable within ten minutes, the relative oxygen uptake dropping from 58.5 to 44.5 per cent In some experiments the fall proved to be far greater, *e.g.* from 50 to about 6 per cent This variety in extent of the effect cannot solely be explained by the presence of a different concentration of histamine in the aerosol Probably the depth of the anaesthesia too is important, as, according to Feldberg and Schulf [1930], in rodents, urethane may weaken the response of the bronchial musculature to histamine

These extensive reactions, however, are always accompanied by a very large decrease in ventilation of the lung to which the histamine is being administered It is hard to say in how far the former effect is influenced by the latter However, proof of the existence of a direct constrictor action of histamine on the vascular bed of the lungs is shown in Table VI by the administration of histamine at 13.44 and at 16.30 In the first case the reaction of the ventilation is very slight, and it is absent in the second, but in both cases a distinct vasoconstriction develops, as shown by the fall in the relative oxygen uptake The weaker response at 16.30 is perhaps due to a greater depth of the anaesthesia When at 14.08 the breathing of the histamine-aerosol is interrupted, the full disappearance of the effect is seen to take about one hour The subsequent partial reversal of the reaction, *i.e.* a slight increase both in blood-flow and in ventilation, should be noted

After the renewed application of histamine at 18.05, neo-antergan 2786 R P (0.1 mg per c c atomiser fluid) was added to the inspired

air According to Ramanamanjary [1944], the antagonistic action of the anti-histaminics against a hypotensive dose of histamine is abolished in rabbits by various anaesthetics, amongst others by urethane. Nevertheless a prompt disappearance of the effects of histamine on the lung circulation has always been observed in our experiments.

Both the inhalation of a histamine-aerosol and a lowering of the alveolar oxygen tension result in a constriction of the vessels of the lung. According to the experience of a number of investigators [Best,

TABLE VI \*

Time		Oxygen uptake		Tidal air		Frequency
		Total c c/min	Per cent R	Total c c	Per cent R	
11 12	R Histamine aerosol	14.3	54.5	14.8	60.1	25
11 45		13.4	58.9	14.9	60.4	26
12 55		13.5	59.3	13.9	60.4	25
13 42		12.8	58.6	14.4	59.0	27
13 44						
13 55	R Aerosol discontinued	12.8	44.5	12.6	48.4	33
14 06		13.4	44.8	14.2	51.4	32
14 08						
14 36	R Histamine aerosol	12.9	48.1	12.8	54.7	31
14 55		13.5	53.3	11.1	50.4	32
15 08		15.6	57.7	13.4	58.2	34
16 28		12.8	62.5	13.0	64.6	30
16 30						
16 40	R Histamine neo antergan aerosol	13.0	58.5	12.2	61.5	34
16 55		12.7	55.1	11.7	59.0	32
17 50		14.5	51.0	10.8	60.2	39
18 01		15.0	52.0	9.9	60.6	39
18 05						
18 19	R Aerosol discontinued	16.0	58.1	9.9	57.6	47
18 38		13.9	62.6	10.2	58.8	42
18 40						
19 35		14.2	60.5	12.4	57.5	33
20 17		13.4	60.4	13.8	58.0	36

\* Both spiographic systems filled with oxygen

Dale, Dudley, and Thorpe, 1927, MacGregor and Peat, 1933, Tarras Wahlberg, 1936, Eichler and Speda, 1940, Eichler, Speda, and Wolff, 1943], the lungs are involved in the metabolism of histamine, and this function seems to depend on the supply of oxygen. This is consistent with the fact that the fermentative breakdown of histamine by the histaminase or diamino-oxydase is essentially an oxidative process in which molecular oxygen fulfils the rôle of a hydrogen acceptor [Zeller, 1942].

With these considerations in mind, various observations have been made to test whether histamine might be involved in the reaction to local hypoxia. They may be summarised in the following way —



(a) At the end of a period of unilateral breathing of a very low oxygen mixture, the histamine content of the nitrogen lung, determined after Code [1937], surpasses that of the oxygen lung by approximately 50 per cent (Table VII). This is, however, only true if the nitrogen lung has breathed nearly pure nitrogen during a couple of hours. In breathing 5 per cent oxygen the difference in histamine content is insignificant. There seems to be no close correlation between the increased histamine content of the nitrogen lung and the degree of vasoconstriction.

(b) In adrenalectomised rabbits, which are known to possess an increased sensitivity, amongst others, to histamine, unilateral ventilation with nitrogen sometimes causes a vasoconstriction within 15 minutes.

TABLE VII

Expt No	Inspiratory air		Histamine content ( $\gamma$ per g)		Difference in per cent
	Duration (hours)	O <sub>2</sub> per cent	Nitrogen lung	Oxygen lung	
22	6½	0.22	14.2	10.4	+37
24	9½	0.46	12.4	7.2	+74
25	9	0.22	10.0	6.3	+59
28	13	4.65	6.1	7.0	-15
35	5½	5.10	23	22	+5

instead of the usual 8 hours [Heemstra, 1948]. The results, however, are complicated by the rapid decline of the general condition of the adrenalectomised animal (progressive fall of the blood-pressure and death) in the course of the experiment. As adrenalectomy not only increases the sensitivity to histamine but also to other substances, it is not permissible to draw a definite conclusion from these experiments.

(c) Anti-histaminics (antergan, neoantergan, 3277 R P) do not suppress the hypoxic vasoconstriction either by application as an aerosol or by subcutaneous injection. The effect of a histamine-aerosol is promptly abolished by these substances.

(d) The administration of a histamine-aerosol does not accelerate the establishment of a hypoxic vasoconstriction. If during the slow development of a hypoxic vasoconstriction a histamine-aerosol is given to the nitrogen lung, a rapid constriction superimposes itself on the gradual one. By discontinuing the supply of histamine the more rapid constriction disappears, while the gradual one may be observed to develop in the ordinary way.

(e) Incidentally it was observed that a rabbit, which did not react on the administration of a histamine-aerosol, exhibited a distinct constriction on breathing nitrogen.

The observations grouped under (a) and (b) are not incompatible with the conception that histamine might be involved in the reaction to local hypoxia, those summarised in (c), (d) and (e), on the other hand, show a different action of histamine and of hypoxia.

This state of affairs resembles that in reactive hyperæmia studied by Folkow, Haeger, and Kahlson [1948]. This reaction, too, is claimed by some workers to result from a liberation of histamine. Anti-histaminics, however, do not exert any influence on the reactive vasodilatation, and there is no correlation between the extremely variable sensitivity to histamine and the magnitude of the reactive hyperæmia. The possibility of other naturally accumulated metabolites dilating the vessels in reactive hyperæmia and causing a constriction of the lung vessels in our experiments cannot be excluded.

#### SUMMARY

By methods described in previous papers the action of various agents on the blood circulation in the lungs has been studied in the living rabbit. Resection of part of the sympathetic trunk, including the upper thoracic ganglia, causes an increase of blood-flow through the lung. Vagotomy is without effect.

Some evidence is produced that carbon dioxide might exert a local vasoconstrictor action. If so, both the development of the effect and its disappearance must be fairly rapid.

In the living rabbit an adrenaline-aerosol produces a distinct constriction of the lung-vessels accompanied by a slight decrease in ventilation.

The effect of acetylcholine-aerosol proves to be a weak vasodilatation, which on the addition of physostigmine changes into a strong vasoconstriction accompanied by a decrease in ventilation. These latter effects are abolished by atropine. A large dose of choline has been found to exert a slight constrictor action.

The inhalation of histamine-aerosol causes a very pronounced narrowing of the lung-vessels, which at a high dose is accompanied by a great diminution of the ventilation. Anti-histaminics (neo antergan) rapidly abolish the effects of the histamine-aerosol.

The adaptation of the calibre of the lung-vessels to the alveolar oxygen tension is not mediated by extrapulmonary nerves. Neither adrenaline, acetylcholine nor histamine seem to be involved in the mechanism of its production. The histamine content of the nitrogen lung generally surpasses that of the oxygen lung by approximately 50 per cent.

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SPONTANEOUS INTRACORPUSCULAR INACTIVATION AND  
REACTIVATION OF HÆMOGLOBIN By G FEGLER<sup>1</sup>  
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INTRODUCTION

It seems to be well established that inactive forms of hæmoglobin which do not combine with oxygen are present in normal circulating blood. Variable amounts, mostly as methæmoglobin, have been demonstrated in the blood of different species [Ammundsen, 1941, Van Slyke, Hiller, Weisiger and Cruz, 1946, Ramsay, 1944, Von Issekutz, 1939, Prado, 1946]. It is important in defining the conditions under which hæmoglobin inactivation occurs to signify whether this is due, on the one hand, wholly or in part to the formation of methæmoglobin or, on the other, to the production of some other inactive form. In this paper the terms "inactive or inactivated hæmoglobin" will be used to denote that the nature of the inactive form has not been experimentally determined. Whenever hæmoglobin inactivation has been shown to be due to methæmoglobin production, the term "methæmoglobin formation" will be used.

It has been shown that methæmoglobin is gradually formed in whole blood and hæmoglobin solutions kept *in vitro*. Neil and Hastings [1925] have shown that spontaneous oxidation of hæmoglobin in solutions largely depends upon a partial deoxygenation of the solutions under test. Brooks [1932, 1935], who confirmed this effect of partial deoxygenation, discovered that pH values of the solutions below the physiological range and temperatures in the neighbourhood of 30° C enhanced the methæmoglobin formation. The fact that blood circulating in active tissues also encounters a low pH and a low partial pressure of oxygen suggests that these conditions may favour inactivation of hæmoglobin (methæmoglobin formation) in the red cells of circulating blood. It is known, however, that one of the conditions preventing the accumulation of methæmoglobin in blood is a constant intracellular reduction of methæmoglobin by glucose which is a process catalysed by an enzyme system [Warburg, Kubowitz, and Christian, 1930, Wendel, 1932, Cox and Wendel, 1942, Kiese, 1946, Gutman, Jandorf, and Bodansky, 1947, Gibson, 1948].

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These results suggest that the concentration of methemoglobin in the blood is determined by the equilibrium between the conditions governing its intracellular formation and reduction

The aim of the investigation to be described was to carry out preliminary experiments to test this hypothesis. In order to obtain changing conditions *in vitro* closely simulating those which the circulating blood encounters in the living tissues, a technique was developed for the exposure of red blood cell suspensions to appropriate gas mixtures for different periods of time. Horse red blood cells were used in the experiments. The determinations of the degree of inactivation were carried out by gasometric and photometric methods and accordingly the results of experiments will be described in two separate sections

## I SPONTANEOUS INACTIVATION OF HEMOGLOBIN DETERMINED GASOMETRICALLY

### *Methods*

Horse blood collected from the jugular vein was used in these experiments. Heparin B D H or Liquemin Roche (1000 units per 100 ml) served as an anticoagulant. The experiments started usually 2-4 hours after bleeding, and in a few only, blood kept at 6° C for two days was used. Red blood corpuscles were separated by centrifuging, the plasma discarded and replaced by saline. The centrifuging was repeated, saline discarded, and cells were resuspended to make 35-40 vols p c in a medium containing 4/5 of the M/5 phosphate buffer and 1/5 of saline. For the experiments in which the suspensions were subsequently exposed to nitrogen, the pH of the buffering solutions was 7.0-7.20. In experiments in which a mixture of nitrogen and carbon dioxide was used, the pH of the buffer solution varied from 7.30 to 7.40. The suspension was then aerated for one hour at room temperature and the initial capacity for oxygen or carbon monoxide was determined in the Van Slyke-Neil apparatus.

A sample of 5 ml of the suspension was introduced into a 'glass rod' tonometer [Fegler and Banister, 1946], filled by nitrogen or nitrogen and carbon dioxide (5-8 p c) and exposed at 37.5°-38° C for different periods of time (2-15 minutes). At the end of the exposure period a sample of 1 ml was drawn out from the tonometer and the capacity for oxygen or carbon monoxide was determined.

Two methods of determination of oxygen capacity were used. One was similar to Sendroy's [1931] with shaking of the 1 ml sample diluted with saline in the Van Slyke apparatus chamber filled with air or oxygen, or oxygen + CO<sub>2</sub>, in the second method the "glass rod" tonometer was used. The whole suspension after the exposure to N<sub>2</sub> or N<sub>2</sub> + CO<sub>2</sub> was withdrawn from the tonometer and transferred to another one filled

with air or oxygen and exposed at room temperature or at 37.5°–38° C for 15–25 minutes. The contents of oxygen found were corrected for physically dissolved oxygen according to data given by Sendroy, Dillon, and Van Slyke [1934].

For determinations of carbon monoxide capacity, suspensions were saturated with carbon monoxide in the Van Slyke apparatus chamber.

TABLE I.—THE INACTIVATION OF HEMOGLOBIN IN HORSE RBC SUSPENSIONS EXPOSED TO NITROGEN

Expt No	Conditions of desaturation and oxygen capacity determinations	pH	Initial specific oxygen capacity ml O <sub>2</sub> /100 g Hb	p c decrease in specific capacity after the exposure to nitrogen for			
				2 min	5 min	10 min	15 min
55	Desat N <sub>2</sub> -38° C		1 341	3 4			
66	Satur O <sub>2</sub> -38° C		1 337	4 7	5 6		
	Desat N <sub>2</sub> -38° C						
	Satur Air-13° C		1 340		4 4	5 3	
67	Desat N <sub>2</sub> -38° C						
	Satur O <sub>2</sub> -13° C						
68	Desat N <sub>2</sub> -38° C	6 96	1 337		3 4	4 7	
	Satur O <sub>2</sub> -14° C						
69	Desat N <sub>2</sub> -38° C		1 340	2 3	6 7	11 4	11 8
	Satur Air-15° C						
70	Desat N <sub>2</sub> -38° C	7 20	1 340		3 3	6 3	6 8
	Satur O <sub>2</sub> -38° C						
71	Desat N <sub>2</sub> -38° C	6 99	1 340		3 6	8 2	
	Satur O <sub>2</sub> -38° C						
72	Desat N <sub>2</sub> -38° C	7 23	1 339	0 8	5 9	5 4	
	Satur O <sub>2</sub> -38° C						
75	Desat N <sub>2</sub> -38° C		1 340		4 1		
	Satur Air-15° C						
103	Desat N <sub>2</sub> -38° C		1 341		5 0	2 6	
	Satur Air-18.5° C						
110	Desat N <sub>2</sub> -38° C		1 323		2 7	1 3	
	Satur O <sub>2</sub> -18° C						
111	Desat N <sub>2</sub> -38° C		1 336	4 0	5 8	4 8	5 5
	Satur O <sub>2</sub> -18° C						
118	Desat N <sub>2</sub> -38° C	7 26	1 326	5 0	6 6	9 0	
	Satur O <sub>2</sub> -18° C						
	Mean			3 5	4 8	5 9	8 0

and analysed according to Van Slyke, Hiller, Weisger, and Cruz' method [1946] (Due to technical difficulties in obtaining the "ferri-cyanide syringe" recommended by the authors, the solution of ferri-cyanide was evacuated before each analysis) In some experiments the saturation with carbon monoxide was carried out after the exposure period in the same tonometer to which carbon monoxide was added (approximately 60 ml to the 300 ml capacity tonometer), and the

suspension was exposed to it for a period of 5-6 minutes. Then the sample was withdrawn and analysed on carbon monoxide content.

The hæmoglobin concentration was determined in each sample by Stadie's [1920] cyanmethæmoglobin method modified by Wu [1922], using the Hilger-Spekke photo-electric absorptiometer and the green (No. 5) filter. The specific capacity was calculated from vols p.c. of  $O_2$  or CO given by gas analysis and from the hæmoglobin concentration. This was read from a calibration curve obtained by diluting the standard solution of blood in which total hæmoglobin was determined by carbon monoxide capacity method [Van Slyke, Hiller, Weisiger, and Cruz [1946]].

The percentage decrease in specific capacity (ml  $O_2$ /1 g hæmoglobin) was used in the course of these investigations as a measure of the extent of inactivation [presumably methæmoglobin formation].

The pH of the suspensions was measured occasionally, using the portable Cambridge Instrument Co. pH meter and the dip type calomel-glass electrode system.

### *Results*

The results of the series of experiments with exposure of red blood corpuscles (r b c) suspensions (pH 7.00-7.20) to  $N_2$  (Table I) seem to indicate that a definite partial inactivation of hæmoglobin develops after a 2-15-minute period of desaturation. The extent of inactivation varied in different experiments. In some experiments, however, it was possible to carry out determinations of oxygen capacity on the same suspension after exposure to  $N_2$  for at least three different periods of time. The results when plotted against time fell on a smooth curve, thus excluding a gross error. The average percentage of inactivation, calculated from all the data, plotted against time of desaturation also gave a definite curve, which shows that the growth of inactivation decreased with the increase of the time of exposure and therefore with the degree of desaturation (fig. 1, curve No. 1).

In a second series of experiments (Table II) the effect of exposure to  $N_2 + 5-8$  p.c.  $CO_2$  on the r b c suspensions in saline-phosphate buffer, pH 7.30-7.40, was investigated. After the exposure the capacity for oxygen or carbon monoxide was estimated as described above.

These experiments gave results only partially similar to those obtained with exposure to  $N_2$ . Approximately the same rate of inactivation was obtained for exposures lasting up to 5 minutes. When, however, a longer period of exposure was used, for instance 10-15 minutes, reactivation of hæmoglobin became apparent.

This is illustrated by U-shaped curve (fig. 1, curve No. 2) representing the relation between time of exposure and the corresponding



TABLE II THE INACTIVATION OF HEMOGLOBIN IN HORSE RED BLOOD CELL SUSPENSIONS EXPOSED TO NITROGEN-CARBON DIOXIDE MIXTURES

Expt No	Conditions of desaturation and oxygen capacity determination	pH before and after complete saturation in $N_2 + CO_2$	Initial specific oxygen capacity ml $O_2/100$ g Hb	p c decrease in specific capacity after exposure to $N_2 + CO_2$ for				Remarks
				3 min	5 min	10 min	15 min	
38	Desat $N_2 + CO_2$ -38° C Sat $O_2 + CO_2$ -21° C		1 337		5 3	4 7	0 0	$O_2$ capacity by Sendroy's method, $CO_2$ p c in $N_2 + CO_2$ = 8 0
53	"		1 332		1 9			" " " "
82	"		1 348		4 2			" " " "
83	"		1 335	3 1	5 0			" " " "
90	Desat $N_2 + CO_2$ -38° C Sat $O_2 + CO_2$ -38° C	7 29-7 13	1 333		4 7			" " " "
100	Desat $N_2 + CO_2$ -38° C Sat Air-18 5° C	7 42-7 15	1 340		4 0	4 6		$O_2$ capacity by glass rod tonometer technique
101								" " " "
136	Desat $N_2 + CO_2$ -38° C Sat $CO_2$ -38° C	7 32-7 12	1 340 1 335		4 2 5 2		2 9	" " " "
137			1 364		4 6	6 0		CO capacity by glass rod tonometer technique $CO_2$ p c in $N_2 + CO_2$ = 5 0
138			1 339		2 6			" " " "
140			1 338		3 6	1 5	1 3	" " Ox blood CO capacity by glass rod tonometer technique $CO_2$ p c in $N_2 + CO_2$ = 5 0
144	"		1 338		4 1	4 1	4 5	" " " "
	Mean			3 1	4 1	4 2	2 2	CO capacity by van Slyke-Hiller No 2 method

average value of inactivation. This curve, taken in conjunction with curve 1, suggests that when a suspension of r b c is deoxygenated in the presence of carbon dioxide the process develops in three phases (1) growth of inactivation, (2) equilibrium between inactivation and reactivation, and (3) reactivation prevailing over the inactivation. The

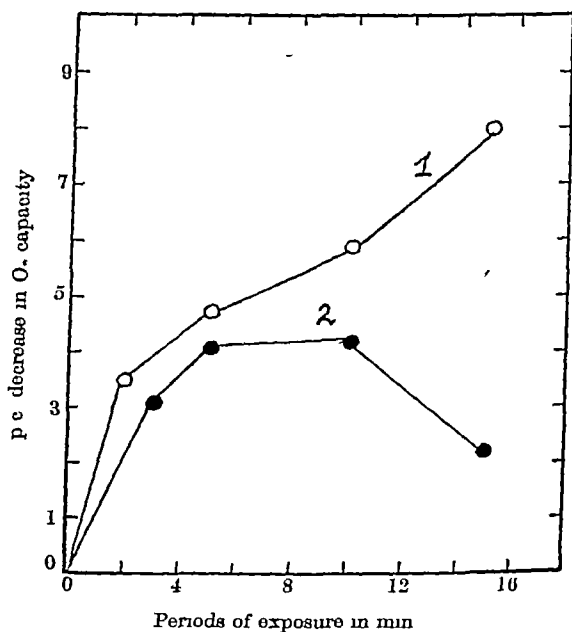


FIG. 1.—The effect of exposure of 5 ml r b c suspension to N<sub>2</sub> (O) and to N<sub>2</sub> + CO<sub>2</sub> (●). Estimation of the p c inactivation of hæmoglobin by gasometric methods.

third phase of the process does not appear at the same time when the suspensions are exposed to nitrogen only. This difference suggests that carbon dioxide may act as an important factor preventing further inactivation.

## II PHOTOMETRIC DETERMINATIONS OF THE SPONTANEOUS INTRA-CORPUSCULAR INACTIVATION OF HÆMOGLOBIN (METHÆMOGLOBIN FORMATION)

### Methods

The preparation of suspensions and the exposure to gas mixtures were carried out using the technique described in the first section. As glucose does not penetrate r b c of the horse [Kosawa, 1914], the

addition of it, except in a few experiments, was usually omitted. More attention was paid to experiments on the influence of exposure to  $N_2 + CO_2$  on inactivation and in only a few experiments  $N_2$  was used. The inactivation of hæmoglobin was also observed in suspensions exposed to air +  $CO_2$  or  $O_2 + CO_2$ .

The extent of inactivation was determined photometrically by the estimation of the percentage of methæmoglobin in the tested suspensions, using a procedure based on the method of Evelyn and Malloy [1938]. This method depends on the fact that NaCN decreases the absorption of light of 630 m $\mu$  wave-length by solutions of methæmoglobin, though not affecting hæmoglobin or oxyhæmoglobin. A Pulfrich photometer with S/61 filter was found most suitable of the methods tried for the photometry. The details were as follows.

The hæmoglobin solution to be tested was prepared by dissolving 0.2 ml. of the rbc suspension in 5 ml. of M/60, pH 6.50 phosphate buffer. Five minutes later, when hæmolysis was completed, pure oxygen was blown through for 15 to 30 seconds. This ensured maximal saturation with oxygen, and also served the purpose of getting rid of any carbon dioxide which the sample contained after being exposed to  $N_2 + CO_2$  or air +  $CO_2$ .

Estimations were made of the extinction coefficient (Pulfrich S/61 filter) of each sample (1 cm. layer) (a) without treatment ( $K_1$ ), (b) after addition of 0.05 ml. of neutralised 5 p.c. NaCN solution ( $K_2$ ), and (c) after further addition of 0.05 ml. of 4 p.c.  $K_3Fe(CN)_6$  solution ( $K_3$ ).

From these readings the proportions of the hæmoglobin, initially present as methæmoglobin, could be evaluated, using a calibration chart prepared from known mixtures. The measurement of  $K_3$  is essential, because the effect of NaCN (measured by  $K_1 - K_2$ ) depends not only on the amount of methæmoglobin present but also on the total quantity of pigment.

In some experiments of this section the pH was measured at the end of different periods of exposure to  $N_2 + CO_2$ . To allow the determinations to be made on small volumes of the suspension, an arrangement constructed on the principle of Stadie's electrode was used. It consisted of two tubes connected by a 3-way tap. One of the tubes was filled with saturated solution of KCl and served as a container for the calomel electrode, the second one served as a container for the glass electrode (Marconi's narrow type). Three ml. of the suspension placed into this container made a sufficiently high column of the liquid to prevent any marked loss of carbon dioxide from its deeper layers during the determination. These measurements of pH were carried out with Marconi's portable pH meter. The system was checked with buffer solutions of known pH and was found to give satisfactory results.

## RESULTS

1 *Effect of the Exposure of Red Blood Corpuscle Suspensions to  $N_2$  and  $N_2 + CO_2$* 

The results of these experiments are collected in Table III. They show relatively large variations, which may have been caused by many factors, including technical errors. Thus the concentration of CO in the gas phase varied from 5 to 7 p c and there was also about 1 p c of  $O_2$  present in commercially supplied cylinders of  $N_2$  and  $N_2 + CO_2$ . The gasometric experiments described in the preceding section suggested

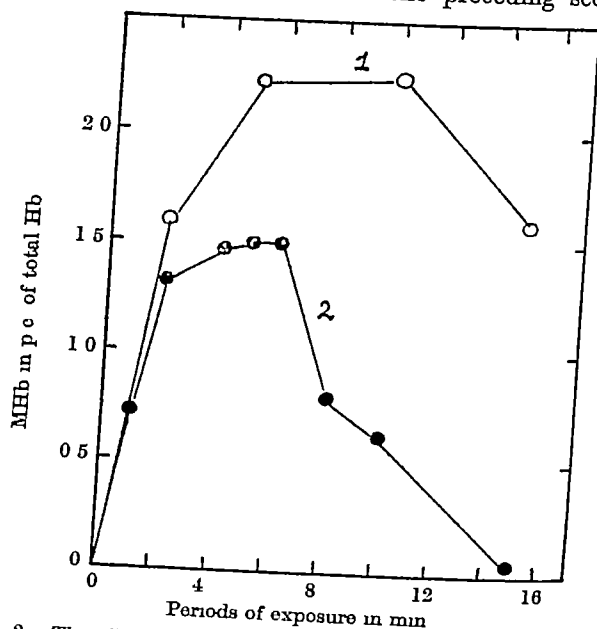


FIG. 2.—The effect of exposure of 5 ml r b c suspension to  $N_2$  (O) and  $N_2 + CO_2$  (●) measured photometrically

that a certain degree of deoxygenation and  $CO_2$  saturation may influence the inactivation and reactivation processes, and it was possible that the variations in partial pressure of carbon dioxide and oxygen in the gas phase could indirectly affect the results of this series of experiments. This suggestion is supported by those particular experiments of this series in which the formation and reduction of methæmoglobin were determined on the same suspension after several different periods of exposure. As in the "gasometric" series of experiments here also the results of these more complete experiments were expressed by a smooth curve. Also the average figures when plotted against the time of exposure to  $N_2$  or  $N_2 + CO_2$  gave curves of the same character as those drawn from data of any other complete experiments. This is illustrated by fig. 2.

TABLE III—RESULTS OF PHOTOMETRIC DETERMINATIONS OF METHAEMOGLOBIN

TABLE III —RESULTS OF PHOTOMETRIC DETERMINATIONS OF													Remarks	
Expt No	pH previous to exposure	p.p CO <sub>2</sub> of the gas phase, mm Hg	p c methaemoglobin found before (initial) and after the exposure for											
			Initial	1 min	2 min	3 min	4 min	5 min	6 min	8 min	10 min	15 min		
171	7.35	42.4	0.14		1.2		1.5		1.5		0.80		0.06	Suspension contains 0.1 p c glucose Suspension contains 0.1 p c glucose Exposed to N <sub>2</sub> , p c desat in 2 min = 28 p c
173	7.30	42.2	0.00	0.60					1.5		0.36			
174	7.30	42.7	0.13		1.4	1.1			1.7					
175	7.30	42.5	0.10		1.1									
176	7.30	35.7	0.00		1.1				1.5		0.30	0.10		
181	7.30	35.8	0.30						2.1			0.00		
191	7.30	34.8	0.00											
195	7.30	35.1	0.00		1.5				1.1					
223	7.35	47.0	0.00						1.1			0.00		
223A	7.35	47.0	0.00		1.7				1.1					
224	7.30	47.3	0.00								0.82	0.00		
225	7.30	47.3	0.23		1.4	1.8			2.2		2.4			
229	7.20	0.0	0.00											

TABLE III—RESULTS OF PHOTOMETRIC DETERMINATIONS OF METHHEMOGLOBIN—(continued)

Expt No	pH previous to exposure	p p CO <sub>2</sub> of the gas phase, mm Hg	p c methemoglobin found before (initial) and after the exposure for										Remarks
			Initial	1 min	2 min	3 min	4 min	5 min	6 min	8 min	10 min	15 min	
230	7 20	0 0	0 00					2 6			2 4	2 3	Exposed to N <sub>2</sub>
231	7 30	41 4	0 00				1 4	1 7			0 84		
235	7 30	48 5	0 00	1 1									Contains 0 1 p c glucose
235A	7 30	48 5	0 11	1 4	1 8		1 5				0 33	0 9	
236	7 00	0 0	0 00					2 3					"
237	7 15	0 0	0 00					1 8			1 9		
201	7 38	43 6	0 17	1 5	1 5								"
205	7 45	"	0 17					2 0					
205A	7 45	30 0	0 18					1 5					At end of 10 min exposure pH=7 20
205B	7 45	45 0	0 18					1 1			0 97		
206	7 40	60 0	0 18								0 80		At end of 2 min exposure p c O <sub>2</sub> released = 38 p c CO <sub>2</sub> abs 75 p c pH changes after 2 min exposure pH=7 18 5 min pH=7 12, 10 min pH=7 16
207	7 38	44 5	0 00	0 80	1 3			1 5					
Average for N <sub>2</sub> + CO <sub>2</sub> exposure			0 08	0 70	1 35	1 45	1 47	1 53	1 5	0 80	0 63	0 05	
Average for N <sub>2</sub> exposure			0 00		1 60			2 23					

The photometric determinations of the spontaneous inactivation (methæmoglobin formation) confirmed the results of gasometric experiments carried out in similar conditions. As regards the effect of exposure to  $N_2 + CO_2$  (fig 2, curve 2), a similar U-shaped curve was obtained showing the phases of (1) formation of methæmoglobin, of (2) an equilibrium between the formation and reduction of it, and (3) a reactivation process which markedly prevailed over the formation.

The effect of exposure to  $N_2$  (fig 2, curve 1) differs from that of exposure to  $N_2 + CO_2$  in that the concentrations of methæmoglobin found at different periods of exposure to  $N_2$  are higher than in  $N_2 + CO_2$  experiments. Also the "equilibrium" phase lasts longer, so that at 15 minutes of exposure to  $N_2$  the "reactivation" phase, though already visible, is much less marked than in  $N_2 + CO_2$  experiments. This last phase in the " $N_2$ " curve is, however, better shown in these experiments than in the corresponding gasometric experiments. Also the extent of methæmoglobin formation in the " $N_2$ " experiments as well as in " $N_2 + CO_2$ " experiments found by photometric determinations is less pronounced than in gasometric series. A qualitatively similar difference was found previously by Van Slyke, Hiller, Weisiger and Cruz [1946].

The U-shaped curve obtained in experiments with exposure to  $N_2 + CO_2$  suggested that  $CO_2$  might be important as a factor preventing the accumulation of methæmoglobin. Yet it should be noted that a decreased pH within the r b c produced either by a suitable buffer or by uptake of  $CO_2$  is important for the formation of methæmoglobin. Many determinations (gasometric and photometric) carried out in the course of the present work on suspensions exposed to  $N_2$  for 1-15 minutes showed that there was no measurable formation of methæmoglobin provided the pH was kept constant at 7.30-7.40. This indicated that deoxygenation alone without change in pH was not a major factor of intracorpuseular methæmoglobin formation.

The pH of a suspension exposed to  $N_2 + CO_2$  usually decreased from 7.4-7.3 to 7.2-7.1 respectively. It was then rather surprising that suspensions in which pH was kept at the same level (pH 7.2-7.1) by a buffer, when exposed to  $N_2$  behaved in a different manner than those exposed to  $N_2 + CO_2$ . An explanation for this difference may be the specific effect of  $CO_2$ . This gas can penetrate easily into r b c and cause there a sudden decrease in pH, thus influencing the release of  $O_2$  more effectively than when the suspension kept at constant pH 7.2-7.1 is exposed to  $N_2$ . Since  $CO_2$  increases the rate of  $HbO_2$  dissociation, and also, as might be expected from the results of Ramsay [1946] and Neil and Hastings [1925], more complete deoxygenation favours the disappearance of methæmoglobin, it is possible that the extent of methæmoglobin formation and reduction may depend not only on partial pressure of carbon dioxide in  $N_2 + CO_2$  mixtures but also on the time of exposure.

A scatter diagram was therefore constructed (fig 3) on which concentrations of methæmoglobin were plotted against corresponding p p CO<sub>2</sub> used in exposures to N<sub>2</sub> + CO<sub>2</sub>. The results of exposure for 2 and 5 minutes (Table III) were selected for the diagram. The scatter diagram shows that at 2-minute periods of exposure the concentrations of methæmoglobin tend to increase with the rising p p CO<sub>2</sub>, while an opposite relation exists at 5-minute periods of exposure. The correlation

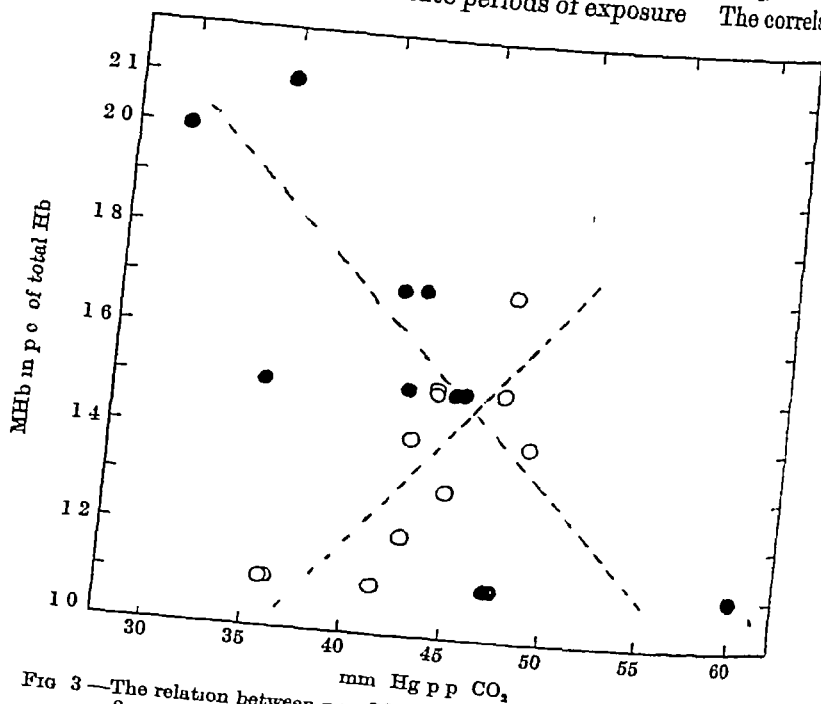


FIG 3—The relation between p p CO<sub>2</sub> and p c methæmoglobin formed at 2 minute (O) and at 5 minute (●) periods of exposure

coefficients were calculated for both cases and were found to be sufficiently significant according to Fisher's [1941] "P" table

This treatment of results suggests that in the initial stage of exposure when the extent of CO<sub>2</sub> uptake is greater than the corresponding degree of deoxygenation, the CO<sub>2</sub> uptake stimulates the formation of methæmoglobin. When however, in the later stage of exposure, deoxygenation has already reached a certain critical degree, the reduction of methæmoglobin becomes apparent in spite of the further uptake of CO

## 2 The Methæmoglobin Formation in Oxygenated Suspension

Completely oxygenated blood is said to be free of methæmoglobin because no reduced hæmoglobin (necessary for the formation of methæmoglobin) would be present



Experiments described below show, however, that even complete oxygenation could not prevent some methæmoglobin formation provided temperature and pH were favourable

In one experiment aerated suspensions of r b c in M/5 phosphate buffers of different pH were kept at 38° C for one hour and then the concentration of methæmoglobin in each was determined photometrically. The results of this experiment are tabulated below

pH	p c methæmoglobin
7.40	0.09
7.35	0.00
7.30	0.09
7.20	0.90
7.08	1.24
7.00	1.34
6.60	1.50

Further, in a series of experiments the exposure for different periods of time (5-15 minutes) to a mixture of air or oxygen with carbon dioxide of 5 ml suspension placed in a "glass rod" tonometer was carried out at 38° C. At the end of the exposure methæmoglobin was determined photometrically. In some of these experiments the change in pH after the exposure was also measured. The results are given in Table IV

TABLE IV — METHÆMOGLOBIN FORMATION IN SUSPENSIONS OF R.B.C. EXPOSED TO AIR+CO<sub>2</sub> AND O<sub>2</sub>+CO<sub>2</sub> (5 ML. SAMPLE 38° C)

Expt No	Exposed to	Percentage methæmoglobin					
		Initial	5 min	7 min	10 min	12 min	15 min
259	Air + 50 mm p p CO <sub>2</sub>	0			1.6		
263	Air + 50 mm p p CO <sub>2</sub>	0	1.0		1.8		1.3
263A	O <sub>2</sub> + 60 mm p p CO <sub>2</sub>	0			1.9		
264	O <sub>2</sub> + 55 cm p p CO <sub>2</sub>	0	1.0				1.8
265	"	0	1.1				
265A	Air, pH of susp = 7.08	0.1			1.2		
271	O <sub>2</sub> + 57 mm p p CO <sub>2</sub>	0	1.1		1.4		1.6
302	Air + 40 mm p p CO <sub>2</sub>	0		2.1			2.2
302A	O <sub>2</sub> + 57 mm p p CO <sub>2</sub>	pH = 7.38 0 pH = 7.40				2.7 pH = 6.8	pH + 7.18

From the results of these experiments it may be concluded that, even at the highest possible saturation with oxygen, methæmoglobin might be formed within the r b c when in the presence of CO<sub>2</sub> or a suitably decreased pH at 38° C

### 3 Experiments with the Interchange of the Gas Phase

In these experiments the exposure of the test sample of rbc suspension was divided into 3 stages

- (1) Exposure for 10 minutes to air containing  $\text{CO}_2$  at 28-40 mm Hg partial pressure
- (2) Transfer of suspension treated in (1) to the atmosphere of  $\text{N}_2$  with  $\text{CO}_2$  at 50-60 mm Hg p p for two periods of time 2-5 minutes and 10 minutes respectively
- (3) Repeated exposure of the suspension treated in (2) to air +  $\text{CO}_2$  as in (1)

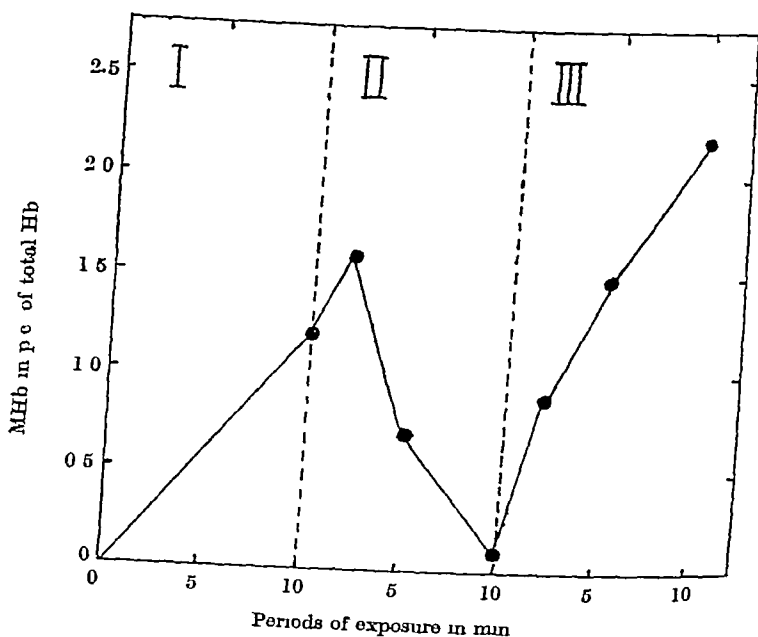


FIG 4 —Effect of exposure of the same sample of rbc suspension I, to air +  $\text{CO}_2$  II to  $\text{N}_2$  +  $\text{CO}_2$  III, again to air +  $\text{CO}_2$

All exposures were made in the "glass rod" tonometers at  $38^\circ \text{C}$ , and at each stage the concentration of methaemoglobin was determined photometrically

These experiments formed an attempt to imitate the changing conditions of  $\text{O}_2$  and  $\text{CO}_2$  saturation in blood as it flowed through the lungs and tissues

The results of this series of experiments are given in Table V and illustrated in fig 4. It might be inferred from them that the main site of methaemoglobin formation is the lung. The conditions which

the blood meets in the tissues favour the reduction of preformed methæmoglobin, though there may be a short initial period of its further formation

TABLE V—RESULTS OF EXPERIMENTS WITH THE INTERCHANGE OF THE GAS PHASE

Expt No	I Oxygenation period Exposure to air + CO <sub>2</sub> in minutes		II Deoxygenation period Exposure to N <sub>2</sub> + CO <sub>2</sub> in minutes						III Oxygenation period Exposure to Air + CO <sub>2</sub> in minutes			
	pp CO <sub>2</sub>	10	pp CO <sub>2</sub>	2	3	5	10	pp CO <sub>2</sub>	2	5	10	
275	28.2	1.3	80.5	1.4			0.0	28.2		1.0		
276	40.0	1.6	50.0	1.5			0.3	40.0	0.9			
277	40.0	1.3	50.0	0.9			0.2	40.0		1.6		
278	28.6	0.8	61.2	1.4			0.0	28.6			2.7	
279	28.7	1.3	61.7	2.1		0.7	0.0	28.7		1.4	1.8	
282	28.5	0.9	50.0		2.4		0.3	28.5		1.9		
Average	32.3	1.2	55.6	1.6		0.7	0.1	32.3	0.9	1.5	2.3	

## DISCUSSION

Experimental results reported in this paper indicate that when horse red blood cells are subjected to pH of 7.2–7.1, and to oxygen at certain critical levels, inactivation of hæmoglobin develops in a comparatively short time. Should, however, the oxygen saturation drop below about 30 p.c., a reactivation becomes apparent, eventually a complete disappearance of preformed methæmoglobin may occur if the degree of deoxygenation becomes sufficiently pronounced. Partial deoxygenation seems to create the right conditions rather for reactivation than for inactivation of hæmoglobin within the r.b.c. This suggestion is supported by the experiments in which methæmoglobin formation was obtained in suspensions of r.b.c. exposed even to pure oxygen provided the intracellular pH was simultaneously decreased. It is not excluded, therefore, that the conditions of a decreased affinity for oxygen such as produced by a decreased pH may favour the intracellular formation of methæmoglobin in concentrations of the range found in these experiments.

Further, mention should be made of the possible inhibitory influence of high oxygen tensions on the reactivation of hæmoglobin.

The importance of the intracellular pH changes for the formation and that of deoxygenation for the reduction of methæmoglobin was illustrated by fig. 3. The scatter diagram on this figure shows that in the conditions of our experiments the initial phase results from the carbon dioxide uptake, which is a faster process than the release of oxygen, so that a considerable drop in intracellular pH occurs before

the deoxygenation has reached a degree favourable for the reactions leading to methæmoglobin reduction

The essential rôle which deoxygenation plays in reactivation of hæmoglobin may also be supported by the following treatment of the results based on Brooks' [1932] paper Brooks, working on laked blood, has found that at the constant (decreased) tensions of oxygen,

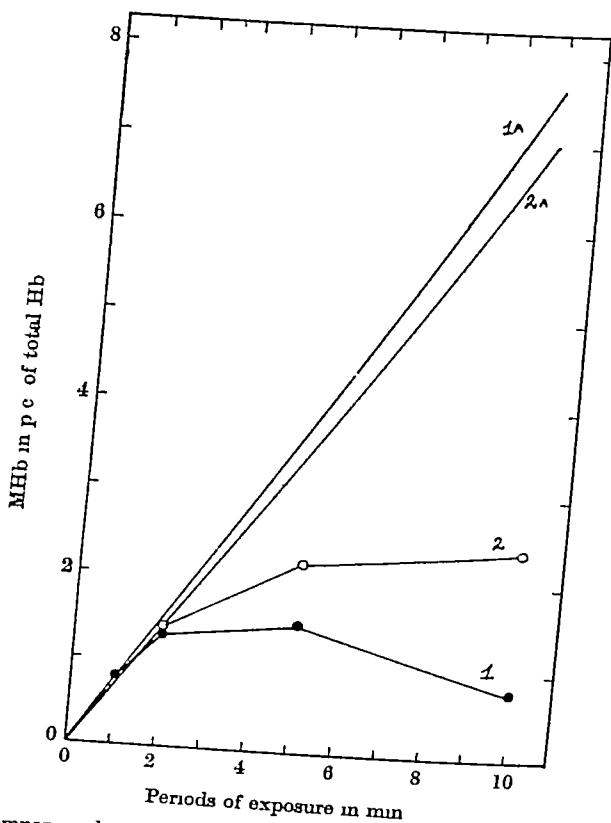


Fig 5 — Comparison between calculated and drawn out-from experimental data curves representing methæmoglobin formation. Curves 1 (experimental) and 1A (theoretical) exposure to  $N_2 + CO_2$ . Curves 2 (experimental) and 2A (theoretical) exposure to  $N_2$ .

methæmoglobin formation is a monomolecular reaction. An attempt was made, therefore, to calculate a complete velocity curve of theoretical methæmoglobin formation, using a velocity coefficient derived from the value of 1-minute exposures of the r b c suspension to  $N_2 + CO_2$  obtained in experiment 297 (Table III). Such a curve (1A) together with the corresponding experimental one (1) are shown in fig 5. The same treatment was applied to data of experiment 229 (Table III), in which percentage methæmoglobin formed at 2-minute periods of exposure to

$N_2$  was used for calculations. The deviation of both experimental curves from the corresponding theoretical curves may serve as a measure of the extent of reduction process counteracting the formation of methæmoglobin within the r b c as the release of oxygen progresses. On the fig 6 percentage differences between experimental and theoretical data were plotted against corresponding percentage of oxygen release and carbon dioxide uptake calculated from data of the same experiments (229 and 297, Table III). An almost straight line relationship between the degree of deoxygenation and the extent of methæmoglobin reduction

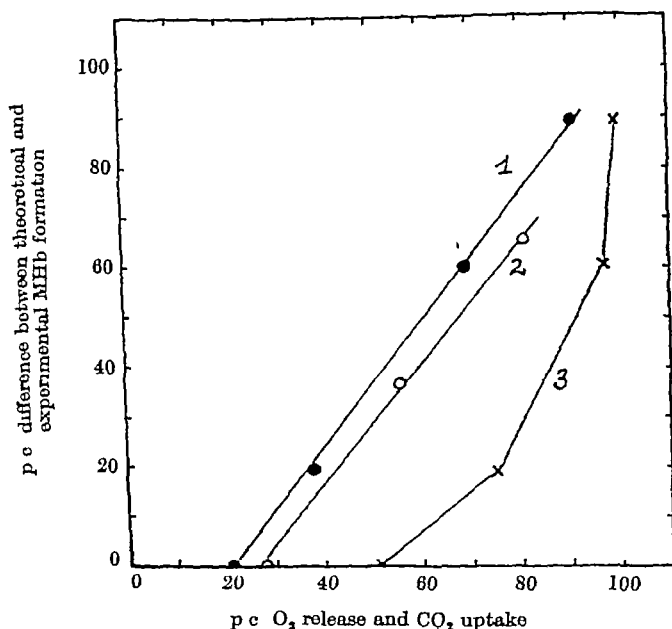


FIG 6—Curve 1 relation between the degree of deoxygenation at the exposure to  $N_2 + CO_2$  and the extent of methæmoglobin reduction (p c difference between theoretical and experimental curves of methæmoglobin formation (see fig 5). Curve 2 the same relation at the exposure to  $N_2$ . Curve 3 relation between the degree of  $CO_2$  uptake and the extent of methæmoglobin reduction

is shown by the curves 1 and 2 on fig 6. The relation between the carbon dioxide uptake plotted against the values of methæmoglobin reduction is shown on the same figure by the curve 3, which suggests that there is a less direct effect of carbon dioxide uptake on the methæmoglobin reduction than that of deoxygenation.

The method used in the experiments described measured the relatively slow exchange which occurs between a liquid and a gas phase. However, as definite relations have been found between the degree of deoxygenation and carbon dioxide uptake and the intracorpuseular inactivation and reactivation of hæmoglobin, it is probable

that similar relations would be found should a method which employs rapid exchange between the liquid phases be used [Hartbridge and Roughton, 1923, 1925, 1926, Dirken and Mook, 1931]

In the light of these considerations, the U shaped curve may be probably regarded as an imitation (though in much longer time) of the events happening when blood comes in contact with tissues. The formation of methæmoglobin, or a further increase in concentration of methæmoglobin already present, may take place in the initial stage of the exposure to higher tension of carbon dioxide, but as the blood loses its oxygen, the formation of methæmoglobin stops and its reduction proceeds. If, depending on conditions of flow through capillaries and on intensity of oxygen consumption and carbon dioxide production, the deoxygenation was very extensive, all inactive hæmoglobin present in arterial blood may be reactivated, so that blood free of methæmoglobin would return to the lungs. There, however, the blood meets again the conditions which favour partial inactivation, which may proceed as long as the blood is under influence of the oxygen at the presence of carbon dioxide. It is suggested, therefore, that in the circulating blood there may be changing conditions, causing a cyclic variation in concentration of inactive hæmoglobin.

The spontaneous methæmoglobin formation has been shown to be very small. What the significance of it may be remains to be investigated. It seems that the changes in oxygen capacity are too small to play any important rôle in decreasing the supply of oxygen to the tissues. But it has been found (author's unpublished experiments) that a small formation of methæmoglobin by appropriate concentrations of sodium nitrite increased the rate of oxygen release, measured by the "slow exchange method," from the r b c suspensions, exposed to  $N_2$  or  $N_2 + CO_2$ . Whether a similar effect would occur when methæmoglobin formation develops spontaneously remains to be investigated.

### SUMMARY

1 The intracorpuseular inactivation of hæmoglobin (presumably methæmoglobin formation) occurred in the suspensions of the horse r b c (pH 7.4-7.3) when these were exposed to gas mixtures of air - CO oxygen +  $CO_2$ , and  $N_2 + CO_2$  for different periods of time (2-15 minutes). Also inactivation occurred in the r b c suspensions in buffer solutions of pH 7.2-7.1 when exposed to air or nitrogen.

2 The reactivation (reduction of methæmoglobin) succeeds the inactivation if the exposure to  $N_2 + CO_2$  or  $N_2$  lasts longer than several minutes. The phenomenon may be expressed by a U-shaped curve showing the phases of inactivation, equilibrium, and reactivation.

3 The extent of reactivation depends on the degree of deoxygenation while the intracellular decrease in pH is a main factor of the inactivation.

- 4 Temperatures in the region of 38° C favour the inactivation as well as the reactivation
- 5 Attempts were made to imitate *in vitro* the changing conditions to which the blood is exposed in the vascular bed by subjecting the suspensions to suitable changes of gas phase
- 6 The results obtained were discussed in relation to *in vivo* conditions

## ACKNOWLEDGMENT

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# INTRACORPUSCULAR METHÆMOGLOBIN FORMATION AND ITS RELATION TO THE RATE OF OXYGEN RELEASE

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## INTRODUCTION

SOME of the author's unpublished experiments carried out in connection with the investigation of the conditions influencing the rate of exchange of oxygen in blood *in vitro* [Fegler and Banister, 1946], suggested that the velocity of oxygen release from partially inactivated ( $\text{NaNO}_2$ ) sheep or ox red blood cells was increased in comparison with the normal cells

In the present paper further experiments on similar lines are reported, the problem being extended to the investigation of the relationship between the spontaneous inactivation ( $\text{HbOH}$  formation) and the release of oxygen from suspensions of horse red blood cells

## 1 PARTIAL INACTIVATION OF HÆMOGLOBIN BY SODIUM NITRITE AND THE RELEASE OF OXYGEN

### *Methods*

Blood of three healthy horses used in these experiments was collected from the jugular vein Heparin B D H (1000 International units per 100 c c) served as an anti-coagulant The suspensions of partially inactivated r b c were prepared in two different ways (1) to 20 ml of the blood, 1-3 ml of 0.1-1.0 p c sodium nitrite solution in 0.9 p c saline were added, the blood being carefully mixed with it, one to three minutes later the poisoned as well as a sample of normal blood was centrifuged, the plasma discarded and the process repeated, the r b c being washed in saline 2-5 times Then the normal (control) and the poisoned r b c were mixed with saline-phosphate buffer solution, pH 7.0-7.3 to make suspensions containing about 40 vol p c red blood cells These suspensions are marked in Table I as " $\text{NaNO}_2$  thoroughly" or "insufficiently washed" (2) A sample of the blood was centrifuged and the plasma discarded and replaced by saline-phosphate buffer

<sup>1</sup> In receipt of a personal grant from the Agricultural Research Council

The suspension was divided into two portions, to one of them saline was added, to the other one an equal volume of  $\text{NaNO}_2$  solution was added, to make a final concentration of 4-8 mg p c. These suspensions are marked in Table I as " $\text{NaNO}_2$  unwashed". Then both samples were placed in flasks of 1 litre capacity, aerated for at least 45 minutes at room temperature, and collected into small Erlenmeyer flasks. The hæmoglobin concentration (Wu-Stadie CNMHB method) or hæmatocrit value was determined in both samples of r b c suspensions to ensure equal conditions.

Samples (5 ml) of the control and the poisoned suspensions were exposed to  $\text{N}_2$  or  $\text{N}_2 + 6-8$  p c  $\text{CO}_2$  in a "glass rod" tonometer, using the technique described by Fegler and Banister [1946].

The time of exposure and the temperature of the bath varied from 1 to 2 minutes and  $16^\circ-38^\circ \text{C}$  respectively. After the exposure the percentage of desaturation was determined in respect of the initial oxygen content (Van Slyke-Neil apparatus), and the coefficient of the velocity of  $\text{O}_2$  release was calculated from the equation

$$K = \frac{2 \cdot 303}{t} \log_{10} \frac{a}{a-x},$$

where  $a = 100$  p c saturation with  $\text{O}_2$ ,  
 $x =$  p c of  $\text{O}_2$  released,  
 $t =$  time of exposure

The concentration of methæmoglobin in the poisoned suspension which had been aerated and kept at room temperature gradually increased during the course of the experiment. This was especially true of the suspensions in which some  $\text{NaNO}_2$  was present in the medium. Since the degree of desaturation in a sample exposed to nitrogen was calculated from the difference between the initial  $\text{O}_2$  content and that found after the exposure, it was essential that the initial  $\text{O}_2$  content was determined simultaneously with the time of exposure. This being technically impossible, the initial  $\text{O}_2$  content had therefore to be controlled several times in the course of the experiment, and the correct initial value exactly corresponding to the time of exposure had to be found from a curve drawn out of the results of these determinations. All experiments in which the "initial"  $\text{O}_2$  content was determined only once before the exposure were discarded, and the results of only those experiments with one initial value were accepted in which cells were at least 5 times washed after they were poisoned.

The same procedure was adopted for the determination of the initial  $\text{O}_2$  content in the control samples, because many preliminary observations showed that the hæmoglobin became gradually inactivated in them although at a slower rate than in the poisoned samples. This happened especially in cells suspended in a medium of  $\text{pH} = 7.0$ .

The results of experiments were corrected for the difference in volume p c r b c or hæmoglobin concentration which occurred to a greater or lesser degree between the control and poisoned suspensions. The correction was based on the straight-line relationship existing between the rate of  $O_2$  release and concentration of r b c or hæmoglobin [Fegler and Banister, 1946]

### Results

The results of this series of experiments are given in Table I. They are grouped according to the technique used as described above.

An increase in the velocity coefficient of  $O_2$  release from partially inactivated suspensions was found in all the experiments except one, in which some definite concentration of  $NaNO_2$  was present in the suspension ( $NaNO_2$  unwashed) or in which there had been insufficient washing and some  $NaNO_2$  was probably left. In all experiments in which cells were washed more completely (Group I) no difference in the rate of  $O_2$  release was found between the control and poisoned suspension. It may therefore be assumed that the rate of  $O_2$  release from poisoned suspensions is only increased when conditions are such that further inactivation of hæmoglobin can occur during the exposure to  $N_2$  or  $N_2 + CO_2$ . No increase in  $O_2$  release is found when the degree of inactivation of the hæmoglobin is more or less steadily fixed as in the experiments on " $NaNO_2$  thoroughly washed cells". In this case at pH 7.0 methæmoglobin is probably formed to an equal degree in both of the suspensions, control and poisoned, when exposed to  $N_2$  or  $N_2 + CO_2$  [Fegler, 1948]. In the case of suspensions which contain some  $NaNO_2$  it is possible that the inactivation is increased during the period of deoxygenation to a greater extent than in the control suspensions. This suggestion is supported by one experiment which was carried out for the purpose of finding out the relation between the extent of inactivation by  $NaNO_2$  and the partial pressure of  $O_2$  to which a suspension is exposed. The protocol of this experiment is given below.

Experiment 48 (Table II). Ox r b c aerated suspension (40 V p c r b c) in saline-phosphate buffer pH 7.40 mixture. Five ml samples of the suspension were treated as described in Table II.

The partial pressure of  $O_2$  in the fifth sample (Table II) was roughly calculated from a determination of the degree of desaturation in an additional sample which was shaken in  $N_2$  for 3 minutes. It was found that 60 p c of the  $O_2$  had been released and 40 p c of it was left, which gave a point on a dissociation curve for ox blood at low percentage  $CO_2$ , corresponding to about 20 mm p p  $O_2$ .

In fig. 1 the percentage decrease in  $O_2$  capacity caused by  $NaNO_2$  was plotted against the partial pressure of  $O_2$ , and an almost straight line was obtained indicating a proportionality between the extent of

The suspension was divided into two portions, to one of them saline was added, to the other one an equal volume of  $\text{NaNO}_2$  solution was added, to make a final concentration of 4-8 mg p c. These suspensions are marked in Table I as "NaNO<sub>2</sub> unwashed". Then both samples were placed in flasks of 1 litre capacity, aerated for at least 45 minutes at room temperature, and collected into small Erlenmeyer flasks. The hæmoglobin concentration (Wu-Stadie CNMhb method) or hæmatocrit value was determined in both samples of r b c suspensions to ensure equal conditions.

Samples (5 ml) of the control and the poisoned suspensions were exposed to  $\text{N}_2$  or  $\text{N}_2 + 6-8$  p c  $\text{CO}_2$  in a "glass rod" tonometer, using the technique described by Fegler and Banister [1946].

The time of exposure and the temperature of the bath varied from 1 to 2 minutes and  $16^\circ-38^\circ \text{C}$  respectively. After the exposure the percentage of desaturation was determined in respect of the initial oxygen content (Van Slyke-Neil apparatus), and the coefficient of the velocity of  $\text{O}_2$  release was calculated from the equation

$$K = \frac{2.303}{t} \log_{10} \frac{a}{a-x},$$

where  $a = 100$  p c saturation with  $\text{O}_2$ ,  
 $x =$  p c of  $\text{O}_2$  released,  
 $t =$  time of exposure

The concentration of methæmoglobin in the poisoned suspension which had been aerated and kept at room temperature gradually increased during the course of the experiment. This was especially true of the suspensions in which some  $\text{NaNO}_2$  was present in the medium. Since the degree of desaturation in a sample exposed to nitrogen was calculated from the difference between the initial  $\text{O}_2$  content and that found after the exposure, it was essential that the initial  $\text{O}_2$  content was determined simultaneously with the time of exposure. This being technically impossible, the initial  $\text{O}_2$  content had therefore to be controlled several times in the course of the experiment, and the correct initial value exactly corresponding to the time of exposure had to be found from a curve drawn out of the results of these determinations. All experiments in which the "initial"  $\text{O}_2$  content was determined only once before the exposure were discarded, and the results of only those experiments with one initial value were accepted in which cells were at least 5 times washed after they were poisoned.

The same procedure was adopted for the determination of the initial  $\text{O}_2$  content in the control samples, because many preliminary observations showed that the hæmoglobin became gradually inactivated in them although at a slower rate than in the poisoned samples. This happened especially in cells suspended in a medium of  $\text{pH} = 7.0$ .

TABLE II—EXTENT OF INACTIVATION AFTER EXPOSURE TO  $N_2$  IN R B C SUSPENSIONS SATURATED WITH  $O_2$  AT DIFFERENT PARTIAL PRESSURE

Treatment of the sample in the chamber of the Van Slyke apparatus	Capacity for oxygen, V per cent	Extent of inactivation in per cent of capacity at (1)
(1) 2.5 ml saline + 1 ml. suspension shaken in oxygen for 10 minutes	16.58	
(2) 2.5 ml saline + 1 ml suspension first shaken in nitrogen for 10 minutes and then in oxygen for 10 minutes	16.51	0.4
(3) 2.0 ml saline + 1 ml suspension first shaken in oxygen for 10 minutes, then 0.5 ml 0.2 per cent $NaNO_2$ solution was added and the shaking was carried out for a further 5 minutes	15.94	3.8
(4) 2.0 ml saline + 1 ml suspension first shaken in air for 3 minutes then 0.5 ml 0.2 per cent $NaNO_2$ solution was added, 3 minutes later air was exchanged for oxygen and the shaking was carried out for 10 minutes	15.40	7.1
(5) 2.0 ml saline + 1 ml suspension first shaken in nitrogen for 3 minutes then 0.5 ml 0.2 per cent $NaNO_2$ solution was added, 3 minutes later nitrogen was exchanged for oxygen and the shaking was carried out for 10 minutes	15.22	8.2

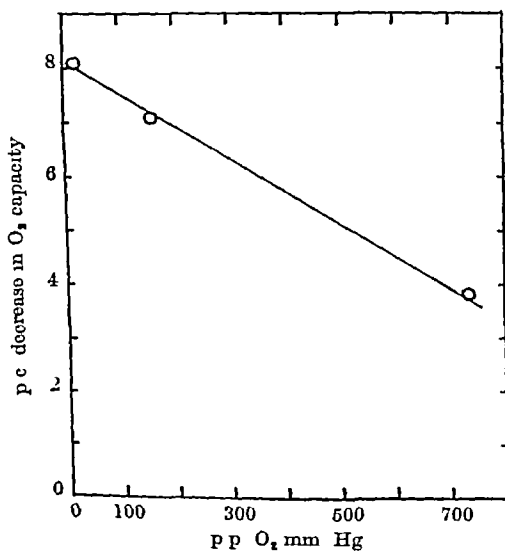
FIG 1—Decrease in  $O_2$  capacity in r b c suspension poisoned by  $NaNO_2$  and exposed to  $N_2$ , air, and  $O_2$

TABLE I—RATE OF O<sub>2</sub> RELEASE FROM HORSE NORMAL RBC SUSPENSIONS AND FROM SUSPENSIONS CONTAINING PARTIALLY INACTIVATED HEMOGLOBIN (NaNO<sub>2</sub>)

FULLY INACTIVATED HÆMOGLOBIN (NaNO<sub>2</sub>)

Expt No	Temp °C	Composition of the gas phase	Control suspensions		Methemoglobin suspensions			K <sub>m</sub> /K <sub>c</sub>	Remarks
			Initial O <sub>2</sub> content, V per cent	Coefficient of O <sub>2</sub> release velocity, K <sub>c</sub>	Initial O <sub>2</sub> content, V per cent	Per cent inactivation	Coefficient of O <sub>2</sub> release velocity, K <sub>m</sub>		
I "NaNO <sub>2</sub> washed" (thoroughly) r b c									
16	25	N <sub>2</sub> + 0 p c CO <sub>2</sub>	20.50	0.1520	16.51	18.9	0.1546	1.017	
148	38	N <sub>2</sub>	19.32	0.2168	18.90	1.8	0.2107	1.000	
151	38	N <sub>2</sub>	18.69	0.2579	18.57	0.63	0.2637	1.022	
II "NaNO <sub>2</sub> washed" (insufficiently) r b c									
24	37	N <sub>2</sub>	19.52	0.3081	13.20	27.0	0.3478	1.129	
25	37	N <sub>2</sub>	19.31	0.2255	14.93	22.3	0.3076	1.364	
27	37	N <sub>2</sub>	20.13	0.2248	18.63	8.0	0.2455	1.092	
65	37	N <sub>2</sub>	19.98	0.1997	18.13	9.2	0.2485	1.244	
III "NaNO <sub>2</sub> unwashed" r b c									
21	20	N <sub>2</sub> + 8 p c CO <sub>2</sub>	17.36	0.1370	15.42	11.5	0.1548	1.22	Final concentration of NaNO <sub>2</sub> , 0.8 mg p c
23	37	N <sub>2</sub>	21.12	0.1891	18.80	11.0	0.2167	1.146	Final concentration of NaNO <sub>2</sub> , 0.4 mg p c
28	37	N <sub>2</sub>	14.97	0.2837	11.20	5.6	0.2807	1.011	Final concentration of NaNO <sub>2</sub> , 0.4 mg p c

### Results

Table III contains the results of this series of experiments

In all experiments of this series  $\text{NaNO}_2$  caused an increase in the rate of  $\text{O}_2$  release

Fig 3, in which the percentage increase in the coefficients of velocity of  $\text{O}_2$  release are plotted against percentage decrease in  $\text{O}_2$  capacity

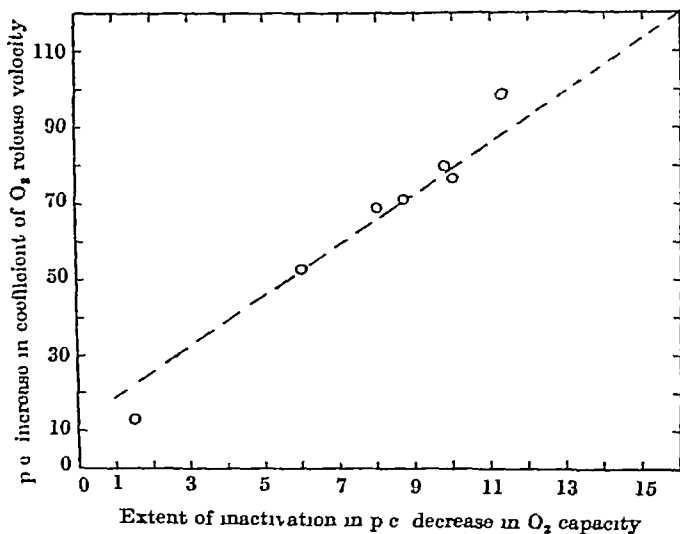


FIG 3—Relation between the extent of inactivation produced by different concentration of  $\text{NaNO}_2$  acting on r b c suspensions during the exposure to  $\text{N}_2$ , and the rate of  $\text{O}_2$  release

caused by  $\text{NaNO}_2$ , shows a very close relationship between these values. It may be roughly calculated that the inactivation of about 1.5 p c of the total hæmoglobin caused by  $\text{NaNO}_2$  during the exposure to  $\text{N}_2$  corresponds to about 10 p c increase in the rate of  $\text{O}_2$  release.

### 3 THE RELEASE OF OXYGEN FROM SUSPENSION OF R B C POISONED BY SODIUM AZIDE

In a series of as yet uncompleted experiments the author has found that sodium azide causes the formation of methæmoglobin. This effect was first suspected when it was observed that aerated suspensions of horse r b c (pH 7.3–7.4) to which  $\text{NaN}_3$  was added (concentration about 0.3 p c) became dark after standing at room temperature for several hours. The darkening was distinctly enhanced at  $37^\circ\text{--}38^\circ\text{C}$ , especially if the poisoned suspension was exposed to  $\text{N}_2 + \text{CO}_2$  (5–6 p c) at this temperature and then re-aerated. It was found that if the r b c of a suspension which had been poisoned and had been left to get dark were thoroughly washed in saline and then examined spectroscopically they

inactivation and the p p  $O_2$ . This means that greater inactivation takes place at a low partial pressure of  $O_2$ .

Fig 2 shows the lack of correlation between the amount of inactivated hæmoglobin present in the sample before exposure to nitrogen and the rate of  $O_2$  release. This is suggestive evidence that the increased velocity of  $O_2$  release from poisoned suspensions depends on a further formation of methæmoglobin.

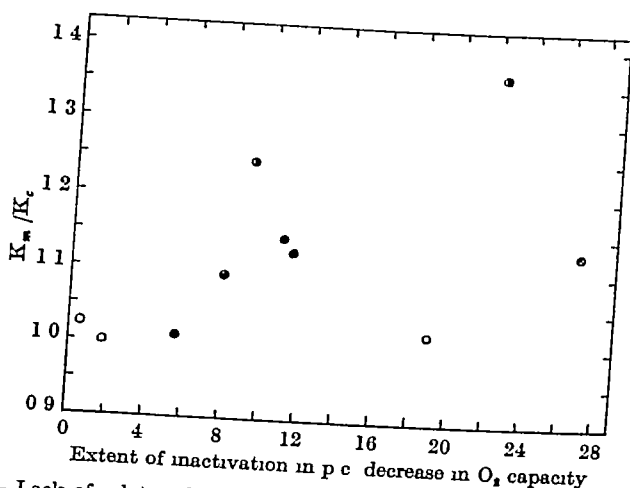


FIG 2—Lack of relation between the degree of inactivation of Hb produced by  $NaNO_2$  in r b c suspensions (before exposure to  $N_2$  or  $N_2 + CO_2$ ) and the increase in the rate of  $O_2$  release. Data taken from Table I.  $\circ$  = Group I,  $\bullet$  = Group III.

## 2 THE EFFECT OF SIMULTANEOUS INACTIVATION BY $NaNO_2$ AND DEOXYGENATION ON THE RATE OF OXYGEN RELEASE

### Methods

The technique used in these experiments was as follows. The horse blood was centrifuged, the plasma discarded, and a suspension (40 V p c r b c) in saline-phosphate buffer pH 7.0–7.4 mixture prepared. The suspension was then aerated at room temperature. Then 0.5 ml of the saline-buffer mixture was introduced into a “glass rod” tonometer followed by 5 ml of the suspension. This was equilibrated with air at  $38^\circ C$  and the initial  $O_2$  capacity at this temperature was determined. The same procedure was repeated with the tonometer filled with nitrogen and containing 0.5 ml of the saline-buffer mixture or 0.5 ml of a solution with  $NaNO_2$ .

At the end of the 1-minute exposure period two 1-ml samples were drawn out into a double-container Ostwald pipette, and the degree of desaturation and the capacity for oxygen (Sendroy's method) were simultaneously determined in two separate Van Slyke apparatus.



showed a typical spectrum with an absorption band at 620–630  $\mu$ . The shadow disappeared after the addition of cyanide solution. It was concluded that a dissociable azide-methæmoglobin compound described by Keilin [1937] was formed in r b c suspensions poisoned by sodium azide. In control suspensions at this pH (7.3–7.4) no appreciable methæmoglobin formation was observed even if they had been exposed to air at room temperature for several hours.  $\text{NaN}_3$  therefore causes the formation of methæmoglobin in conditions in which the spontaneous formation of it is so slow that it could not be measured. As far as it is known to the author, no such previous observation is available. It was known, however, that sodium azide does not react with oxyhæmoglobin nor with hæmoglobin. An indirect effect, possibly through a formation of a methæmoglobin-azide compound resistant to the reduction, or an inhibition of the enzymatic mechanism of methæmoglobin reduction, may therefore be assumed.

In several experiments the formation of  $\text{HN}_3\text{MHb}$  was quantitatively determined in r b c suspensions (pH 7.3–7.4) exposed for different periods of time to  $\text{N}_2 + 5\text{--}6\text{ p c CO}_2$ . The determinations of the degree of inactivation of hæmoglobin were made in one experiment gasometrically and in five experiments photometrically (Pulfrich's photometer, S61 filter). The photometric determinations were based on the above-mentioned observation that aerated suspensions containing  $\text{HN}_3\text{MHb}$  become darker than the control suspensions. The difference in coefficients of extinction were measured (Pulfrich's photometer) before ( $K_1$ ) and after ( $K_2$ ) addition of a drop of the 4 p c  $\text{K}_3\text{Fe}(\text{CN})_6$  solution to a tested solution of  $\text{HbO}_2$  prepared from a suspension poisoned by  $\text{NaN}_3$  (0.3 p c —final concentration) by dilution of 0.4 ml of the suspension in 10 ml of M/60 pH 6.5 phosphate buffer solution. The difference between  $K_2$  and  $K_1$ , maximal when no methæmoglobin was present, became smaller proportionally to the concentration of  $\text{HN}_3\text{MHb}$ . The Pulfrich photometer was accordingly calibrated. The maximal difference (100 p c  $\text{HN}_3\text{MHb}$ –100 p c  $\text{HbO}_2$ ) proved to be large enough (approximately 0.600, depending upon the concentration of the total pigment) to ensure sufficient sensitivity of the method.

Fig. 4 gives the results of these experiments.

The curve 1, fig. 4, drawn out from the mean figures when compared with the curve 2, which represents a U-shaped curve usually obtainable in the same conditions in control experiments [Fegler, 1948], indicates that the formation of  $\text{HN}_3\text{MHb}$  in relation to the time of exposure to  $\text{N}_2 + \text{CO}_2$  is uninterrupted by the reduction process.

In connection with this observation in six experiments the velocity of  $\text{O}_2$  release from suspensions poisoned by  $\text{NaN}_3$  (concentration about 0.3 p c) was determined in comparison with the release of  $\text{O}_2$  in the same conditions (exposure to  $\text{N}_2 + 4.5\text{--}8\text{ p c CO}_2$ ) from the suspensions of normal cells (pH = 7.3–7.4).

TABLE III—THE RATE OF OXYGEN RELEASE DURING SIMULTANEOUS DEOXYGENATION AND INACTIVATION  
SUSPENSIONS OF RBC IN SALINE PHOSPHATE BUFFER MIXTURE, pH = 7.40, EXPOSED TO  $N_2$  AT 38°

Expt No	Control suspensions		Suspensions partially inactivated during deoxygenation			$K_m/K_c$
	Initial oxygen capacity, % per cent	Coefficient of $O_2$ release velocity, $K_c$	Approximate concentra- tion of $NaNO_2$ , mg per cent	Decrease in $O_2$ capacity in % of initial control capacity	Coefficient of $O_2$ release velocity, $K_m$	
115	15.90	0.1839	0.40	4.7	0.2232	1.210
116	14.5	0.1520	0.90	6.0	0.2183	1.436
244	16.92	0.1370	0.80	8.0	0.2070	1.510
245	15.85	0.1683	0.80	10.0	0.2800	1.664
246	18.73	0.1458	0.45	7.3	0.2167	1.485
248	19.03	0.1310	0.40	6.2	0.1863	1.407
"	"	"	0.90	14.4	0.2713	2.070

The relation between the increase in the rate of  $O_2$  release and extent of inactivation is therefore more complicated in this series of experiments than in " $NaNO_2$ " experiments

TABLE IV—THE RATE OF  $O_2$  RELEASE FROM THE NORMAL AND POISONED BY  $NaN_3$  (CONC 0.3 G PER CENT) SUSPENSIONS OF HORSE RBC EXPOSED TO  $N_2 + 4.5-8$  PER CENT  $CO_2$  AT  $38^\circ C$

Expt No	Normal suspension Coefficient of $O_2$ release velocity, $K_c$	Poisoned suspension Coefficient of $O_2$ release velocity, $K_m$	$K_m/K_c$	Remarks
185	0.2256	0.2642	1.170	Exposed to $N_2 + 5$ p c $CO_2$
207	0.2184	0.2623	1.200	Exposed to $N_2 + 6$ p c $CO_2$
314	0.2319	0.2618	1.128	Exposed to $N_2 + 4.5$ p c $CO_2$
315	0.1852	0.2083	1.124	"
318	0.2784	0.3372	1.210	Exposed to $N_2 + 8$ p c $CO_2$
319	0.2637	0.3436	1.303	"

At present it may be concluded that although the mechanism of methæmoglobin formation by  $NaNO_2$  is most probably entirely different from the action of  $NaN_3$ , both of these factors increase the rate of deoxygenation. This would support the suggestion that the formation of methæmoglobin, however it is brought about, is directly related to the increased rate of deoxygenation.

#### 4. INFLUENCE OF CARBON DIOXIDE ON THE FORMATION OF METHÆMOGLOBIN AND ON THE VELOCITY OF $O_2$ RELEASE

It has been found previously [Fegler, 1948] that methæmoglobin is formed in rbc suspensions (pH 7.3-7.4) exposed for a relatively short time to mixtures of carbon dioxide with nitrogen, air, or oxygen. The experiments described below were performed to find out if there is any relationship between the extent of methæmoglobin formation caused by carbon dioxide and the rate of  $O_2$  release from the suspensions exposed to  $N_2 + CO_2$ . The degree of deoxygenation reached after a 5-ml sample of the suspension (pH 7.3-7.4) had been exposed to  $N_2$ , with addition of  $CO_2$  under partial pressure of 32-62 mm Hg, for 30 seconds, was determined gasometrically using the technique described in this paper, and the methæmoglobin formation was estimated photometrically in Pulfrich's photometer [Fegler, 1948].

Results of these experiments are given in Table IV, in all of them an increase was found (13–30 p c) in the rate of  $O_2$  release from poisoned suspensions

More experiments are required to find out if the relation between the

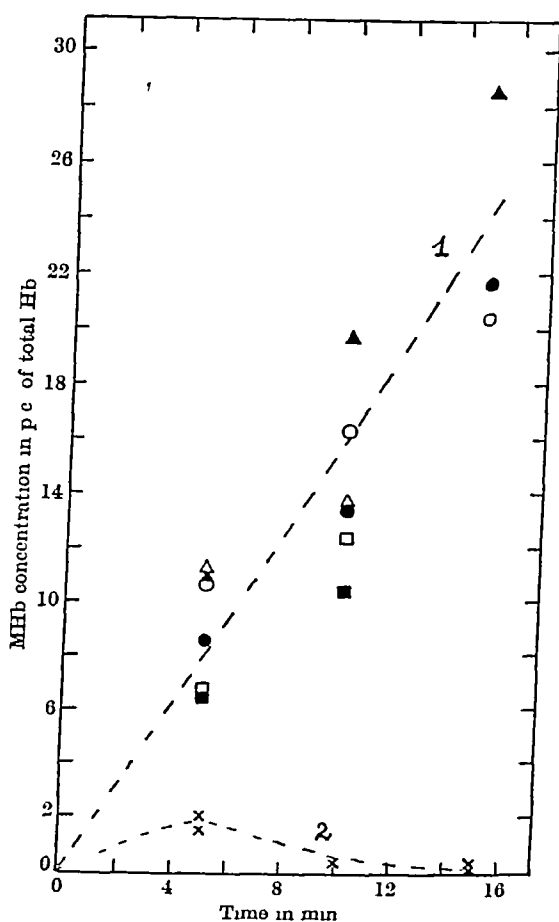


FIG 4—(1) The formation of methemoglobin in r b c suspensions poisoned by  $NaN_3$  (6 experiments) and (2) in normal r b c suspensions (2 experiments) exposed for different periods of time to  $N_2 + 5-6$  p c  $CO_2$  at  $38^\circ C$

increase in the rate of  $O_2$  release and the extent of inactivation caused by  $NaN_3$  is similar to that found in " $NaNO_2$ " experiments. In all experiments with  $NaN_3$  the poisoned suspensions as well as control suspensions were exposed to nitrogen with addition of different concentrations of  $CO_2$ . A variable degree of methemoglobin formation therefore had to be expected also in control suspensions [Fegler, 1918, see also fig 4 in this paper]

TABLE V—THE RATE OF O<sub>2</sub> RELEASE AND THE METHÆMOGLOBIN FORMATION IN HORSE R B C SUSPENSIONS EXPOSED AT 38° C TO NITROGEN, AND TO GAS MIXTURES OF NITROGEN AND CARBON DIOXIDE (32 AND 61 mm Hg p p CO<sub>2</sub>)

Expt No	Exposure for 1 minute to N <sub>2</sub>			Exposure for 30 seconds to N <sub>2</sub> + CO <sub>2</sub> (32 mm Hg)			Exposure for 30 seconds to N <sub>2</sub> + CO <sub>2</sub> (61 mm Hg)		
	MHb in p c of total Hb found after exposure		Coefficient of O <sub>2</sub> release velocity, K <sub>1</sub>	MHb in p c of total Hb found after exposure		Coefficient of O <sub>2</sub> release velocity, K <sub>2</sub>	MHb in p c of total Hb found after exposure		Coefficient of O <sub>2</sub> release velocity, K <sub>3</sub>
305	0 0	0 1832		1 3	0 2071	1 130	3 4	0 2473	1 497
308	0 0	0 1820		0 8	0 1992	1 094	2 3	0 2225	1 223
309	0 0	0 1073		2 9	0 2452	1 243	3 9	0 2778	1 408
310	0 0	0 1911		2 0	0 2493	1 304	3 6	0 3187	1 607
311	0 0	0 1792		2 3	0 2151	1 200	3 6	0 2423	1 352
Mean	0 0	0 1866		1 9	0 2232	1 195	3 4	0 2671	1 431

Table V gives the results of these experiments. In all of them an increase in the rate of  $O_2$  release caused by exposure to two different concentrations of  $CO_2$  corresponded to certain formations of methämoglobin. In fig 5, concentrations of methämoglobin representing the relative rate of its formation under the influence of carbon dioxide

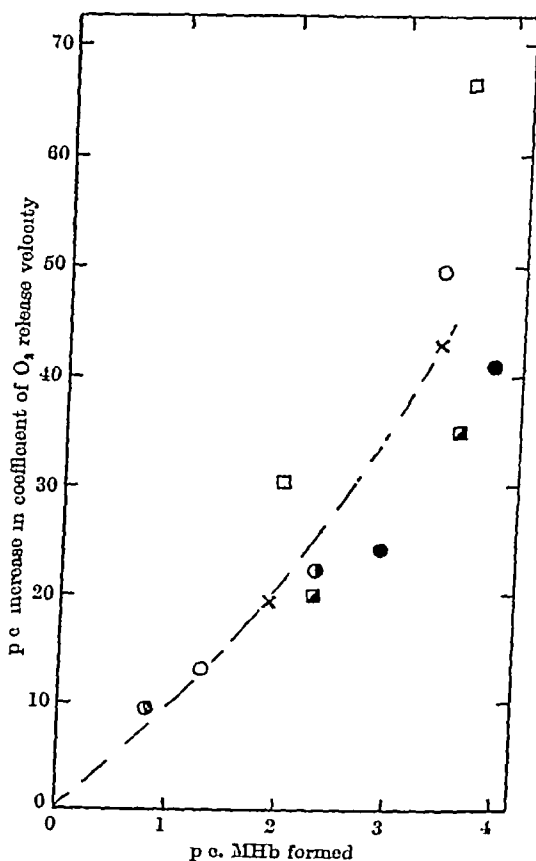


FIG 5—Relation between the extent of methämoglobin formation after 30 seconds of exposure to  $N_2 + CO_2$  (33 and 61 mm Hg p p  $CO_2$ ) at  $38^\circ C$  and the percentage increase in the rate of  $O_2$  release calculated against the rate of  $O_2$  release from r b c suspensions exposed to nitrogen only. Results of 5 different experiments taken from Table V.  $\times$  = mean figures

are plotted against the percentage increase in the velocity of  $O_2$  release calculated in respect of the rate of  $O_2$  release from the suspensions exposed to  $N_2$  only

The figure shows a close relationship between the two values similar to that found in " $NaNO_2$ " experiments

Since it was found in the previous experiments [Fegler, 1948] that methämoglobin is formed in the completely oxygenated suspensions

**O<sub>2</sub> release** This consideration leads to a further suggestion that the well-known effect of carbon dioxide on the release of oxygen might be partially an effect of methæmoglobin formation. More direct evidence is required to give full support to this suggestion.

These considerations are applicable to the conditions of the slow method of determination of the velocity of O<sub>2</sub> release used in our experiments. The mechanism of the increase in O<sub>2</sub> release is in our experiments most probably connected with the change in conditions influencing the diffusion of the oxygen from the liquid into the gas phase. The concentration of r b c and hæmoglobin, and the temperature of exposure, were equal in the control and exposed to N<sub>2</sub> + CO<sub>2</sub> (or poisoned by NaNO<sub>2</sub> and NaN<sub>3</sub>) samples. It is probable, therefore, that an increase in O<sub>2</sub> tension in the liquid phase, caused by the release of an additional volume of oxygen during the methæmoglobin formation, may be the main cause of the increased rate of O<sub>2</sub> diffusion into the gas phase. The only important difference between the conditions in the control and those exposed to N<sub>2</sub> + CO<sub>2</sub> suspensions, is that the CO<sub>2</sub> absorption should rather work in the opposite direction to the described effect, since CO<sub>2</sub> is known to increase the viscosity of a suspension of r b c in plasma or in other medium.

Darling and Roughton [1942] determined the dissociation of oxyhæmoglobin in ox, dog, and human r b c suspensions in which a different degree of inactivation was obtained by the formation of methæmoglobin.

The dissociation curve was shifted to the left in the suspension which contained methæmoglobin, indicating an increase in the affinity of hæmoglobin for oxygen. These findings were confirmed by Lester and Greenberg [1944].

In view of the results of our experiments, it seems reasonable to suggest that the phenomenon discovered by Darling and Roughton is connected with the presence of a relatively fixed concentration of methæmoglobin in their suspensions. Their conditions of experiment are, therefore, not comparable with the conditions of our experiments, in which the concentration of methæmoglobin undergoes changes during the process of deoxygenation.

#### SUMMARY

1 The relative rate of O<sub>2</sub> release from horse r b c suspensions (pH 7.0–7.4) measured with a slow reaction method was investigated in relation to the methæmoglobin formation.

2 A straight-line relationship was found between the relative rate of methæmoglobin formation by sodium nitrite and the increase in the rate of O<sub>2</sub> release.

provided the pH was decreased (exposure to air + CO<sub>2</sub> or O<sub>2</sub> - CO<sub>2</sub>), it seems justifiable to suggest that in the relationship established in the above-described experiments the relative rate of methæmoglobin formation might be a primary factor leading to the increased O<sub>2</sub> release

## DISCUSSION

The results of the experiments described in this paper show that when a suspension of horse r b c is exposed to nitrogen in the presence of a small concentration of sodium nitrite, the resulting degree of deoxygenation is higher than in a control suspension treated in exactly the same way and in the same conditions. On the other hand, no difference in the rate of deoxygenation occurs when poisoned cells have been thoroughly washed, thus fixing the concentration of inactivated hæmoglobin during the exposure. It may therefore be accepted that the process of methæmoglobin formation is responsible for the increase in rate of O<sub>2</sub> release, and that the fixed amount of methæmoglobin in the sample is not important. It might be argued that methæmoglobin formed by NaNO<sub>2</sub> is only a by-product of a direct reaction between NaNO<sub>2</sub> and O<sub>2</sub>Hb at which oxygen is released, and that the sodium nitrite and not the inactivation is a responsible factor in the observed phenomena. This may be true for the case of NaNO<sub>2</sub> action, but it seems more complicated and less understandable in the case of actions of NaN<sub>3</sub> and CO<sub>2</sub>. Although NaN<sub>3</sub> does not react directly with hæmoglobin, it also causes an increase in the rate of deoxygenation. It is most probable that in this case the methæmoglobin formation is a spontaneous process, and the NaN<sub>3</sub> is only responsible for preventing the reduction of the preformed methæmoglobin.

There is no evidence that the higher degree of deoxygenation in NaN<sub>3</sub> poisoned suspension exposed to nitrogen is due to the consumption of oxygen, so it seems to be of some interest that the reactions leading to the formation of methæmoglobin in this case, though different from the reactions of NaNO<sub>2</sub> with hæmoglobin, produce a similar effect as regards the rate of O<sub>2</sub> release.

The experiments with the exposure of r b c suspensions to N<sub>2</sub> - CO in which a spontaneous formation of methæmoglobin is caused by CO have revealed, similarly to "NaNO<sub>2</sub>" experiments, a close interdependence between the extent of methæmoglobin formation and the increase in the rate of O<sub>2</sub> release. Carbon dioxide is the main factor which produces intracorpuseular spontaneous methæmoglobin formation in the conditions of our experiments. This process is not prevented even by complete saturation of the suspension with oxygen [Fegler 1948]. It may therefore be suggested that in the above-mentioned relationship the relative rate of the spontaneous methæmoglobin formation is a primary factor leading to an increase in the rate of



SOME EFFECTS OF AN ACUTE TOXIC SUBSTANCE FORMED  
BY SOLUTION OF METHYL-BIS (B-CHLOROETHYL)  
AMINE IN WATER AFTER INJECTION IN ANIMALS  
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*(Received for publication 10th September 1948)*

THE effect of the nitrogen mustard—methyl-bis (B-chloroethyl) amine—on the skin, by mouth, by inhalation, or by injection in suitable solvents, in animals has been described by Cameron [1943], Cameron, Courtice, and Jones [1947], and a general survey of its biological actions has been recently published by Gilman and Philips [1946]. Briefly, anorexia, vomiting, salivation, blood-stained diarrhoea, reduction in plasma volume, hæmo-concentration, progressive loss of body-weight, peripheral circulatory failure, and death are the usual course of events. Smaller non-lethal doses produce in addition severe damage to the bone-marrow, reflected in the peripheral blood by lymphopenia, granulocytopenia, thrombocytopenia, and moderate anæmia.

Similarly, the hydrochloride of methyl-bis (B-chloroethyl) amine either by mouth or injection in animals has the same toxic effects [Cameron, 1943, Houck, Crawford, Bannon, and Smith, 1947]. During the early work on this substance it was found to be soluble to the extent of 1 g in 100 c.c. of water, and this solution was used for injection. It was apparent, however, that some chemical change occurred after 48 hours in solution as a new acute toxic action was produced when it was injected into animals. Boyland [1946] has described this altered toxicity in mice.

Unlike mustard gas, which becomes non-toxic on standing in water, methyl-bis (B-chloroethyl) amine has been shown by Hanby, Hartley, Powell, and Rydon [1947] firstly to form reaction products including ethylenimonium compounds, and other reactions lead to the formation of dimers, hydroxy-ethylchloroethylamines and diethanolamines. A number of these products have been isolated [Hanby and Rydon, 1947]. The dimer was found to be non-toxic, and Boyland [1946] thinks that the acutely toxic agent is probably the methyl hydroxyethyl ethylenimonium chloride—a quaternary base.

The hydrochloride of methyl-bis (B-chloroethyl) amine under suitable conditions of pH will also form the toxic substance on solution in water.

3 A close relationship was also found between the increase in the rate of  $O_2$  release and the relative rate of the spontaneous methæmoglobin formation caused by exposure of the r b c suspensions to  $N. + CO.$  gas mixtures

4 A methæmoglobin formation uninterrupted by the reduction develops in the suspensions poisoned by sodium azide. The phenomenon is probably caused by a combination between sodium azide and spontaneously formed methæmoglobin [Keilin], this compound being resistant to the intracorpuseular enzymatic reduction of the preformed methæmoglobin. Sodium azide also causes an increase in the rate of  $O_2$  release from r b c suspensions

5 A suggestion is forwarded of a possible part played by the spontaneous methæmoglobin formation as a factor promoting deoxygenation

#### ACKNOWLEDGMENT

I am very grateful to Professor I. de Burgh Daly and Dr. P. Eggleton for their advice and constant interest in these investigations

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different species after subcutaneous injection, divided into these three categories. However, the number of animals used was too small to obtain more than a rough estimate of the minimal lethal dose.

Of the long-legged animals, observation of 8 cats and a study of a cine film gave a general picture of the sequence of events. After a sublethal dose of 4 mg/kg s.c. the onset of restlessness and gradual hypotonia was seen within fifteen minutes. The anti-gravity muscles were most obviously affected, notably those of the neck, back, and forelimbs, with consequent ataxia. In association with this weakness of the neck-muscles there was marked tremor on movement, and hypermetria was seen in the forelimbs. Paresis increased until progression was impossible, but even then passive movements were feebly

TABLE

Species	Number of animals	A	B	C
Dog	2	2 mg/kg		
Cat	8	10 mg/kg	1-4 mg/kg	
Horse	1		2 mg/kg	5 mg/kg
Monkey	3		5 mg/kg	8 mg/kg
Goat	2	10-15 mg/kg		
Rabbit	11	15-25 mg/kg	5 mg/kg	10-15 mg/kg
Guinea pig	4		10 mg/kg	
Rat	6		20 mg/kg	

resisted. After 60 minutes paralysis was almost complete even to the extent of ophthalmoplegia, but the pinna reflex and knee-jerk were never lost. Gradually muscle power returned, and in 3 hours the animals' movements and co-ordination were normal and recovery was uneventful.

With larger doses of 10 mg/kg s.c. the flaccid stage was more quickly reached, respiration failed, and death occurred with final convulsions. In such cases the heart continued beating for a few minutes, and artificial respiration prolonged this for some time. At the stage of complete ophthalmoplegia reflection of light on to the dilated pupil caused blinking of the eyelids, and corneal and conjunctival reflexes were present.

*Decerebrated Cats (8 Preparations)*—Doses of 10 mg/kg i.v. and over reduced the rigidity, but extensor tone was not abolished. The recorded knee-jerk was not significantly impaired. Respiration was at first stimulated, becoming deeper and faster, and then failed.

*Spinal Cats*—One preparation was arranged to record a graphic tracing of the knee-jerks. After injection of 20 mg/kg i.v. there was no effect on this reflex within an hour. In two preparations the

This solution of nitrogen mustard in water is heat stable, and only has the acute toxic effects when injected subcutaneously or intravenously in animals. When given by mouth in doses several times larger than those which cause acute neurological symptoms by injections, animals die from the effects of the nitrogen mustard chiefly on the gastrointestinal tract [Boyland, Foss, and Waters, 1942, Boyland, 1946].

This paper describes this acute toxic action, and it is felt that with the wider use of methyl-bis (B-chloroethyl) amine in man for Hodgkin's disease and carcinoma of the bronchus [Goodman *et al*, 1946, Jacobson *et al*, 1946, Rhoads, 1946, Ap Thomas and Collumbine, 1947], its publication now is opportune.

### MATERIAL AND METHODS

As at the time that these experiments were conducted the nature of the acute toxic agent was not known precisely, the doses referred to in this paper are those of pure methyl-bis (B-chloroethyl) amine dissolved in distilled water in 1 per cent solution for over 48 hours at room temperature.

Eight species of animals were studied—rats, rabbits, guinea pig, goats, dogs, cats, a horse, and monkeys (*Macacus rhesus*). Autopsies were conducted on all animals that died. Cats were used for a fuller analysis, and decerebrated and spinal preparations were also studied. The sequence of events was filmed in a dog, cat, goat, horse, and monkey for more leisureed observation.

### RESULTS

As in the experiments on mice described by Boyland [1946], the general effect in all species was neurotoxic, consisting of partial paralysis combined with muscular inco-ordination and kinetic tremor. The effect varied slightly in the different species and the time of onset, and recovery or death depended on the dose injected.

In general, the effect of different doses by subcutaneous injection can be divided into three categories:

- A Acute neurotoxic symptoms progressing to death from respiratory failure within a few hours
- B Acute neurotoxic symptoms followed by recovery within 1 to 3 hours without sequelæ
- C Acute neurotoxic symptoms followed by apparent recovery in a few hours, but with a fatal termination within 1-6 days from effects of systemic nitrogen mustard poisoning

The following table gives a general comparison of doses in the

dilated pupil caused blinking without any alteration of its size—presumably a reflex *via* the VIIth nerve

The motor branch of the Vth nerve may be partially affected, as the lower jaw tended to sag quite early

Conduction in the VIIth nerve was not obviously disturbed, as the corneal and conjunctival reflexes were retained

Auditory acuity was apparently unimpaired, as long as consciousness persisted. Similarly conduction in nerves IX, X, XI, XII was not materially upset, as swallowing and tongue movements remained normal.

Respiration, as noticed in the horse and dog particularly, was initially stimulated, but finally failed. Consciousness was not lost until just before respiration failed.

It seems reasonable, in cats, to exclude a peripheral motor paresis, even after doses 20–100 times greater than those which caused flaccidity, as there was never complete loss of deep or superficial reflexes, and in the spinal and decerebrated preparations there was no apparent loss of neuromuscular conduction by the peripheral nerve. However, the possibility of reduced conduction through the synapses of the reflex arc in the spinal animal was not excluded.

It was thought that the effect was not unlike that of curare, but this was not substantiated on comparison with this drug. Further, prostigmine had no beneficial effect in the rabbit.

Although, in the decerebrated cat, extensor rigidity was lessened by large doses, extensor tone was never abolished, and the preparation did not revert to the spinal type, as it did by simple section of the cord or after injection of chloralose. Thus impulses from the level of Deiter's nucleus and the cerebellum were not entirely blocked.

On human analogy these effects suggested a cerebellar action, but decerebellation in the cat or dog produces an accentuation of action of all the anti-gravity muscles—a probable release phenomenon.

The predominance of hypotonia contradicts this and suggests a lower motor neurone effect, which however is incomplete and the main site of action may be on the anterior horn cells. The inco-ordination and tremor, so evident in all species, can probably be explained by the hypotonia. The absence of decisive experiments made it impossible to postulate the actual mode and site of action, and crucial experiments to settle the point could not be undertaken at the time that these observations were made.

#### SUMMARY

Water contaminated with nitrogen mustard—methyl-bis (B-chloroethyl) amine—was found to contain a heat stable acutely toxic substance, probably methyl B-hydroxyethyl ethylphosphonate, which

peripheral end of the femoral nerve was stimulated directly, but even after 50 mg/kg i.v. there was no effect on neuromuscular conduction, whereas injection of 2 mg/kg i.v. curare produced immediate and permanent loss of response to stimulation of this nerve

*Autopsies*—These were performed on all animals that succumbed. In those that died from respiratory failure the lungs were congested, but there was no oedema or pleural effusion. The pericardium usually contained a little free fluid, and subendocardial petechial hæmorrhages were seen chiefly in the left ventricle. The spleen in dogs was small and dark and the liver and kidneys congested. The peritoneum contained blood-stained fluid, and the gut was congested with a few scattered hæmorrhages in the jejunum and ileum.

In rabbits that recovered from the acute neurological symptoms and later died at about the 6th day after diarrhoea and salivation, dehydration and considerable loss of weight, the pathological findings were those of nitrogen mustard poisoning [Cameron, 1943].

*Oral Administration* of large doses of 1 per cent solution of nitrogen mustard. A cat weighing 3.175 kg. was given this solution 100 mg/kg by stomach tube. It showed no signs of the neurotoxic effect, but developed diarrhoea and vomiting in 60 minutes and died on the 19th day from systemic nitrogen mustard poisoning.

Similarly two goats were given 100 mg/kg by stomach tube without any neurotoxic symptoms. One died after 27 hours and the other after 4 days with all the pathological lesions of nitrogen mustard poisoning.

## DISCUSSION

The predominant effect observed in intact animals after injection of this toxic solution was rapid and progressive muscular hypotonia, but even in a flaccid condition the activity of the reflexes was maintained until just before death. Associated with this hypotonia was considerable muscular inco-ordination and kinetic tremor.

This loss of motor power was well marked in all animals, with consequent disturbance of postural reflexes and extensor tone, best seen in the long-legged animals. Even in advanced stages of hypotonia, however, some degree of resistance to passive movement was retained, and weak purposive and volitional movements were evident until just before death.

In addition there were disturbances of muscles supplied by the cranial nerves. Ophthalmoplegia was seen especially with the bigger doses at an advanced stage of muscular hypotonia. The IIIrd nerve palsy was complete except that ptosis was not seen in the cat or dog whereas it was observed in the horse, monkey, and rabbit. Conduction in the IIInd nerve was not apparently affected, as light shone on the

# PULMONARY VASOMOTOR FIBRES IN THE SYMPATHETIC CHAIN AND ITS ASSOCIATED GANGLIA IN THE DOG

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(Received for publication 4th August 1948)

SYMPATHETIC pulmonary vasomotor fibres most probably issue from spinal segments T<sub>2</sub>-T<sub>7</sub> [Bradford and Dean, 1889, 1894, François-Franck, 1895 *a*, 1895 *b*, Plumier, 1904, 1905] The further course of these fibres has in the past been investigated in isolated perfused lungs, because it is necessary to eliminate cardiac effects which result from stimulation of the cardiac nerves accompanying the pulmonary vasomotor nerves In such preparations both constrictor and dilator responses of the pulmonary vascular bed have been obtained An analysis of these experiments is to be found in the reviews by Daly [1933, 1936], in which full references are given The vasomotor responses to stimulation of the pulmonary nerves in isolated perfused lungs are weak and transient, but may be improved by simultaneous perfusion of the bronchial circulation which maintains their blood-supply in their intrapulmonary course and in the region of the lung hilus A further investigation of pulmonary vasomotor nerve activity has now been carried out using the perfused living animal (P L A) preparation described by Daly, Elsdon, Hebb, Ludany, and Petrovskaja [1942] Our object has been to study the effects produced on the pulmonary vascular bed by stimulation of the sympathetic chain, the *ansa subclavia*, the stellate ganglion (St G) and its branches, the middle cervical ganglion (M C G) and its caudal branches, and the thoracic vasosympathetic nerves (T V S) In confirmation of others, pulmonary vasomotor fibres have been demonstrated in all these nerves, and in addition cell stations for fibres in the upper thoracic sympathetic chain have been discovered in the St G and M C G The fine sympathetic fibres which issue from the chain at levels T<sub>2</sub>-T<sub>4</sub> and proceed medially to the lung hilus [Ionescu and Enachescu, 1928] have not been examined

<sup>1</sup> Holding a Training Grant from the Medical Research Council

When injected subcutaneously into experimental animals an acute neurotoxic syndrome was observed. The essential features of this were rapidly progressive muscular hypotonia, inco-ordination and tremor, followed later by ophthalmoplegia and finally paralysis of respiration.

Ingestion of this solution did not produce this picture at all, but on the contrary was followed more gradually by symptoms of systemic nitrogen mustard poisoning.

With smaller toxic doses complete recovery sometimes occurred after exhibition of hypotonia for some hours, but sometimes death occurred several days later, again from systemic nitrogen mustard poisoning.

From admittedly incomplete evidence it is suggested that the action of this interesting poison may be on the anterior horn cells.

I am indebted to Professors Lovatt Evans, Gaddum, Cameron, Boyland, and Liddell for their advice, and to the Chief Scientific Officer, Ministry of Supply, for permission to publish.

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being in contact with surrounding tissues, so that it was not possible to stimulate any structure other than the nerve under test. In effect the nerves and electrodes were airborne. When this was impracticable, the nerves and electrodes were shielded from the surrounding tissues by a rubber sheet and cotton-wool. If current spread occurred, it was readily recognised by spasmodic contractions of the thoracic muscles. When rib movements were being recorded, these contractions caused a marked and abrupt displacement of the recording lever which lasted for the duration of the stimulus, and the tidal-air tracing also showed a sharp displacement due to mechanical pressure on the lungs. Such effects could be eliminated by reorientation of the electrodes. Experiments will be described demonstrating that current spread, even when deliberately introduced by excitation of muscle adjacent to the pulmonary nerve-fibres, could not of itself elicit pulmonary vascular responses.

In earlier experiments various types of shielded electrodes were used. They were difficult to apply in the confined space available without damage to the lungs or to the nerve itself. We discarded their use in favour of the method described above.

In some experiments in which the rami communicantes and the vertebral nerves were intact, we obtained some evidence of reflex respiratory effects following stimulation of the sympathetic chain and stellate ganglia, but such effects were absent when the rami were cut. This is in agreement with the observation of others that the upper thoracic sympathetic nerves contain sensory fibres which exert a control over the respiratory rhythm [Craigie, 1921, Cromer, Ivy, and Young, 1933, Fegler, 1933]. We performed no experiments to trace the origin and course of these fibres.

As a rule we had no difficulty in distinguishing reflex phenomena from the effects of current spread. In contradistinction to the abrupt displacement of the lever recording rib movements due to current spread, rib movements of reflex origin were reflected as slight or moderate changes in frequency and amplitude of the lever. We regarded such changes as of reflex origin only if they appeared as a result of stimulating the chain when both the nerve and electrodes were airborne.

Excitation of the sympathetic chain or stellate ganglia was not followed by significant changes in the tidal air, so we assumed that in these conditions bronchomotor responses did not cause passive effects on the pulmonary vascular bed. Even so, some preparations were kept fully atropinised by the repeated addition of atropine sulphate to the blood (2-8 mg in 2-4 litres of blood). In these the effects of stimulation were not significantly different from those obtained in non-atropinised preparations. On the other hand, stimulation of the MCG and TVS nerves often caused bronchoconstriction, and this necessitated atropinisation of the preparation.

## METHODS

The perfused living animal (dog) was prepared and the responses were recorded as described in the original paper [Daly *et al*, 1942]. In a few experiments the left lung perfusion method devised by Daly and Duke [1948] was used.

In some experiments horse blood instead of dog blood was employed as the perfusate [Daly and Weatherall, 1945], and no difference in the vasomotor responses was detected.

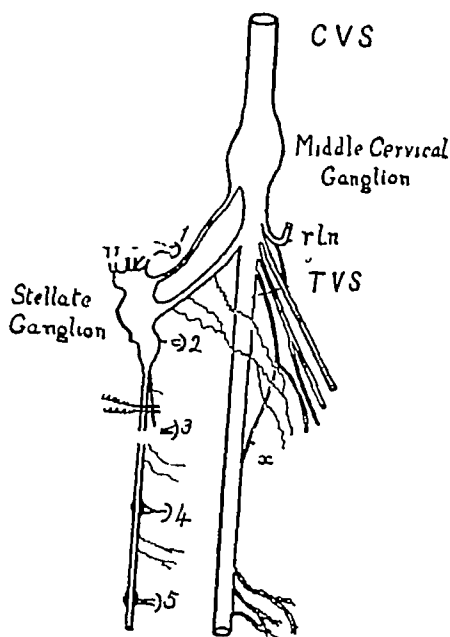


FIG 1 —Diagram of nerve supply to heart and lungs See text

*Stimulation of Nerves*—We used a C F Palmer (London) Ltd induction coil with 4 volts in the primary circuit, or a Ritchie (1944) square wave stimulator in which the voltage, duration, and frequency of the current are under independent control.

For stimulation, the sympathetic chain was sectioned between the 3rd and 4th or between the 4th and 5th thoracic ganglia, while some or all of the rami communicantes cephalad to the section were divided. Platinum wire electrodes were placed on the interganglionic portion immediately cephalad to the section (fig 1). The freed length of chain could be prepared without major disturbance to the lungs. In some experiments the vertebral nerves and the rami connecting the St G with the lower cervical nerves were also divided. Both phrenic nerves were either cut or crushed.

The electrodes were usually applied to the nerves without their



*Cooling of Nerves*—In order to reduce their conductivity, nerves were sometimes placed in contact with a glass tube through which there was continuous flow of iced salt solution. Water at 40° C was passed through the tube to bring the nerves back to normal temperature.

*Recording of Pulmonary Vascular Responses*—Two methods for measuring the pulmonary vascular responses to nerve stimulation were used. In the first, the lungs were perfused at constant blood inflow and the pulmonary arterial pressure was recorded. In the second, the lungs were perfused at a constant pressure, and *changes* in the blood inflow to the lungs as well as the total pulmonary outflow were recorded by a modification of the method used by Alcock, Berry, and Daly [1935]. The arrangement of the apparatus used in the second method is diagrammatically represented in fig 2, *a*. The output side of the pulmonary pump is connected to the pulmonary artery (P A) and to a vertical tube (*a*), the upper end of which terminates in a funnel 30 cm at its greatest diameter. The blood output of the pump is adjusted so that during perfusion of the lungs blood spills over the sides of the funnel and passes down the wide bore tube (*b*), to be returned to the blood reservoir (R A res) from which the pulmonary pump draws blood. The height of the funnel is set 25–30 cm above the lungs. The amount of blood spilling over is measured with a device described by Gaddum [1929]. An electromagnet (1) is operated by a circuit which is intermittently made and broken by contacts on a rotating disc fixed to the shaft of a synchronous electric motor. The current flows for a period of four seconds and during that time operates the lever (*l*) so that the thin rubber tube (*x*) is closed. The blood overflowing down *b* then rises towards the glass bulb (B), and after reaching the horizontal level of *b* starts to move the piston of the P A overflow recorder. At the end of four seconds the circuit is broken and the lever released. The blood in the glass bulb then empties into the reservoir, and the piston returns to the base line. The release period occupies three and one-half seconds. In practice the pulmonary pump is usually set so that the pulmonary overflow reaches about half-way up to the glass bulb B. An increased vascular resistance to blood inflow and thus a diminished blood inflow will be shown by an increase in the height of the excursions of the recorder. The recorder measures *changes* in blood inflow to the lungs at a constant P A p. It does not measure total blood inflow.

The minute volume outflow of blood is obtained by inserting a thin rubber tube (*y*) in the connexions which carry away the blood from the left auricle (L.A). This too is intermittently closed and opened by an electromagnet (2), and the blood entering the glass bulb during a period of five seconds is measured by a second piston recorder. In this case the blood is returned to the left auricle reservoir (L A res). These recording devices are conveniently inserted into the perfusion system of

the P L A originally described by Daly *et al* [1942] and depicted in fig 2 of that paper

An artificial circulation was set up to test the accuracy of these recorders, consisting of "systemic" and "pulmonary" Dale-Schuster pumps connected by rubber tubing to external circuits, the resistances of which gave inflow pressures of approximately 120 mm Hg and 25 cm H<sub>2</sub>O respectively with each pump delivering 120 c c H<sub>2</sub>O per

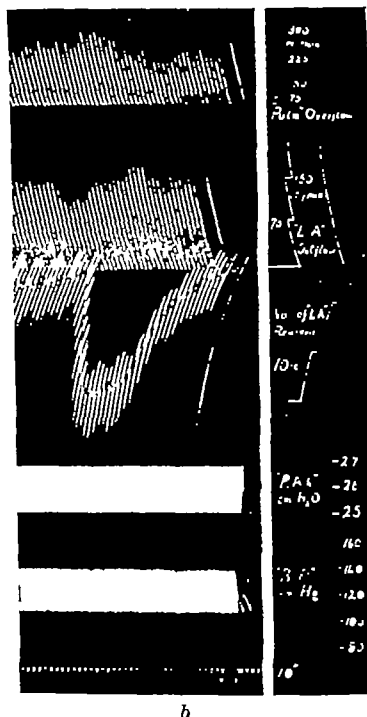
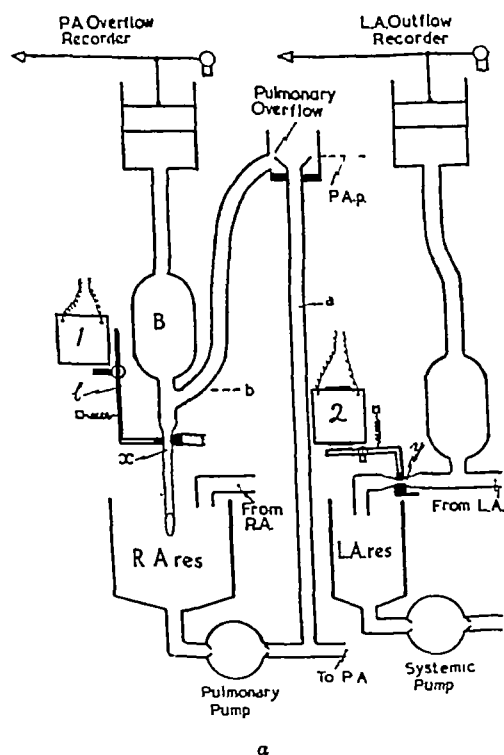


FIG 2—*a* Diagram of apparatus used for measuring blood flow changes to and from the lungs

*b* Records of apparatus test Half natural size For explanation see text

minute In addition, the change in blood-volume of the reservoir (L A res) into which the "L A outflow" emptied was recorded

Fig 2 *b*, represents the results of one test Each excursion of the pulmonary overflow and "L A" outflow recorders represents the flow during a period of four seconds The tracing shows that a temporary constriction of the "pulmonary arterial" tubing increases the "pulmonary" overflow by 28 c c per minute and produces a corresponding reduction in the "L A" outflow The volume of the "L A" reservoir is reduced at the approximate rate of 14 c c in half a minute, which is the expected rate of change With few exceptions

the piston recorders were calibrated after each experiment. A correction has to be made for the volume of blood collecting in tube *b*, this has to be added to that in the glass bulb *B* to give the total "overflow"

*Respiratory Centre Activity*—The movements of the open thorax were recorded by a lever writing on the kymograph paper. The short arm of the lever was attached by a thread to the edge of the split sternum.

## RESULTS

### *Effects of Electrical Stimulation*

*The Sympathetic Chain*—With the chain cut between  $T_3$  and  $T_4$  (or between  $T_4$  and  $T_5$ ) ganglia, stimulation of the cephalad end of the chain will excite ascending fibres issuing from segment  $T_3$  (or  $T_4$ ) and

TABLE I—PULMONARY VASCULAR RESPONSES TO NERVE STIMULATION IN THE PERFUSED LIVING ANIMAL PREPARATION

	Pressor	Diphasic	Depressor	Total
R sympathetic chain	9	0	0	9
L sympathetic chain	5	0	1	6
R St G	8	0	0	8
L St G	2	0	2	4
R.M.C.G.	12	2	0	14
L.M.C.G.	4	2	3	9
R.T.V.S.	9	1	0	10
L.T.V.S.	4	1	1	6
Right side =	38	3	0 =	41
Left side =	15	3	7 =	25
	53	6	7 =	66

R =right, L =left, M.C.G. =middle cervical ganglion, St G =stellate ganglion, T.V.S. =thoracic vagosympathetic nerve

The numerals denote the number of P.L.A. preparations in which each response was observed. The number of tests exceeded 300.

from those segments more caudally situated. These may be pre-ganglionic fibres or ascending post-ganglionic fibres derived from cell stations in the chain ganglia caudal to the third (or fourth) thoracic ganglion.

The predominant response to stimulation of the chain was an increase in the resistance of the pulmonary vascular bed (Table I). This was shown either by a rise in pulmonary arterial pressure (P.A.p.) when the lungs were perfused at constant blood inflow, or by a diminution in total pulmonary inflow and outflow when the lungs were perfused

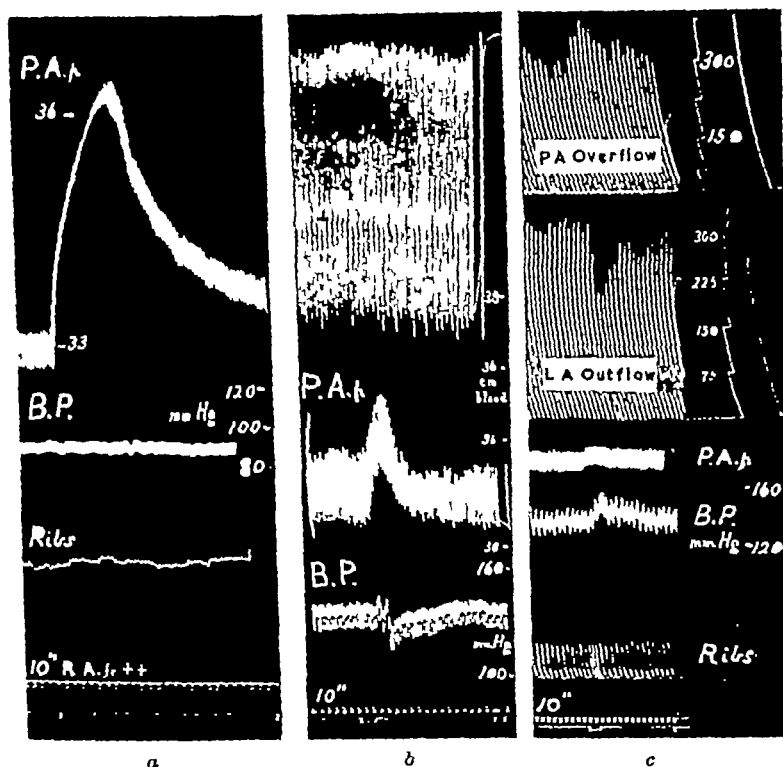


FIG 3—*a* P L A 69 Dog, ♀, 6.7 kg, perfused with horse blood at constant blood inflow to both circulations. Positive pressure ventilation of lungs. Both CVS cut. Duration of perfusion = 3 hr 52 min. Stimulation of L sympathetic chain, coil distance 6 cm. The respiratory centre was inactive, and the oscillations on the "ribs" tracing are due to artificial ventilation of the lungs.

*b* P L A 80 Dog, ♀, 5.9 kg, perfused with "mixed" dog blood at constant blood inflow to both circulations. Negative pressure ventilation. R CVS intact, L CVS cut. Duration of perfusion = 2 hr 45 min. Stimulation of R stellate ganglion at 15 volts 10 msec duration and 50 cycles per second. Atropine sulph 2.0 mg 135 min previously.

*c* P L A 88 Dog, ♀, 8.7 kg, perfused with horse blood at constant blood inflow to the systemic circulation and at constant pulmonary arterial pressure. Positive pressure ventilation. Both CVS ligated. An increase in the excursions of the upper tracing (P.A. overflow) denotes a diminished inflow of blood to the lungs. The calibration figures indicate c.c./min. Duration of perfusion = 4 hr 52 min. Stimulation of the L sympathetic chain at 5/0 5/50.

P A p = pulmonary arterial pressure (cm of blood), B P = systemic arterial pressure (mm Hg), ribs = rib movements, T A = tidal air, inspiration downwards, R A fr = frequency of right auricle.

at constant P A p (fig 3) In one experiment only stimulation of the sympathetic chain caused a pulmonary depressor response This will be described later in detail

In order to maintain responses over a period of some hours we found it desirable to use the smallest strength of stimulus which would give a clearly measurable P A p change The stimulus was applied for ten to fifteen seconds at ten-minute intervals to give P A p changes of 5 to 10 per cent of its initial value (fig 5) This meant that the stimulus had to be increased towards the end of the experiment Using the Palmer coil the stimulus ranged from 14 to 3 cm coil distance, whereas

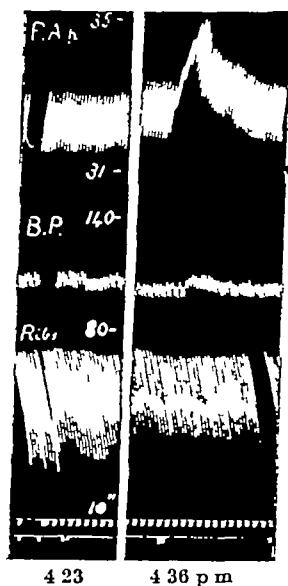


FIG 4 P L A 82 Dog, ♀ 5.4 kg, perfused with "mixed" dog blood at constant blood inflow to both circulations Positive pressure ventilation Both C.V.S. intact Duration of perfusion 1 hr 26 min

4 23 p.m Stimulation of R. *longus colli* muscle at T<sub>2</sub> level, 5/1/50

4 36 p.m Stimulation of R. sympathetic chain 5/1/50

with the Ritchie stimulator the voltage of the stimulus ranged from 5 to 15 The adjustment of the electrodes on the chain generally produced a response Such a response and that due to the first electrical stimulus might be larger than those subsequently elicited by electrical stimulation

In the experiment of fig 4, a stimulus directly applied to the *longus colli* muscle at a distance of  $\frac{1}{2}$  cm from the chain did not give rise to P.A.p. effects, although current spread to surrounding tissues was evident This showed that P.A.p. responses we had obtained were not due to stimulation of adjacent structures, and this was confirmed in other experiments

It was pointed out by Daly and Hebb [1942] that pulmonary pressor



responses to nerve stimulation in isolated lungs perfused through both the pulmonary and bronchial arteries could be explained on the basis of true pulmonary vaso-constriction or of -dilation of the bronchial blood-vessels transferring more blood from the systemic to the pulmonary circulation. The pulmonary pressor responses here described, however, were shown to be due to pulmonary vasoconstriction since they could

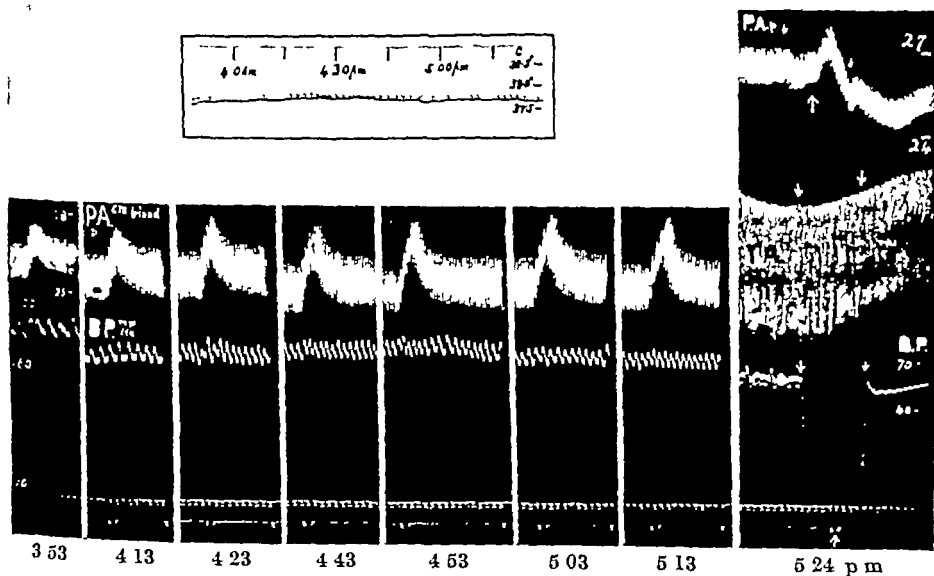


FIG 5—PLA 66 Dog, ♀, 8.6 kg, perfused with "mixed" dog blood at constant blood inflow to both circulations. Positive pressure ventilation. Both CVS cut. Duration of perfusion at 3.53 p.m. = 1 hr 2 min

3.19 p.m. Atropine sulph 2.0 mg added to blood

3.53–5.13 p.m. Series of stimulations of R sympathetic chain, coil distance 5 cm

5.24 p.m. Negative pressure ventilation.

Between arrows ↓↓ the systemic pump was switched off

At arrow ↑ stimulation of R sympathetic chain, c.d. 5 cm

The smaller tracing shows the rectal temperature (upper) and the blood temperature (lower) in the left auricle reservoir. Taken with resistance thermometers and a thread recorder (Cambridge Scientific Instrument Co.)

be obtained at zero systemic arterial pressure (systemic blood pump "off"), during which the transfer of blood from the bronchial to the pulmonary circulation would be stopped. In 7 tests we have only once failed to find during zero systemic blood-pressure a pulmonary response to nerve stimulation similar to that obtained when the systemic pump was sustaining the blood-pressure (fig 5). It was concluded, therefore, that the PAp response to sympathetic nerve stimulation was due in these experiments to constriction of the pulmonary blood-vessels and not to a dilatation of vessels transferring blood from the bronchial to the pulmonary circulation.

at constant P A p (fig 3) In one experiment only stimulation of the sympathetic chain caused a pulmonary depressor response This will be described later in detail

In order to maintain responses over a period of some hours we found it desirable to use the smallest strength of stimulus which would give a clearly measurable P A p change The stimulus was applied for ten to fifteen seconds at ten-minute intervals to give P A p changes of 5 to 10 per cent of its initial value (fig 5) This meant that the stimulus had to be increased towards the end of the experiment Using the Palmer coil the stimulus ranged from 14 to 3 cm coil distance, whereas

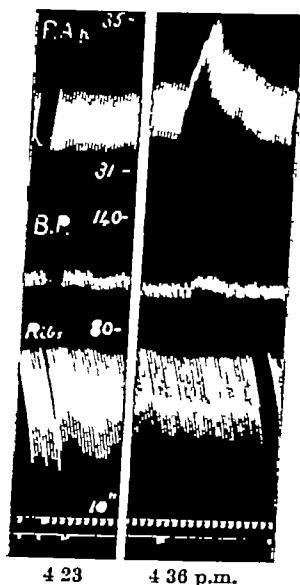


FIG 4 P.L.A 82 Dog, ♀, 5 4 kg, perfused with "mixed" dog blood at constant blood inflow to both circulations Positive pressure ventilation Both C.V.S intact Duration of perfusion 1 hr 26 min

4 23 p.m Stimulation of R *longus coll.* muscle at T<sub>3</sub> level, 5/1/50

4 36 p.m Stimulation of R sympathetic chain, 5/1/50

with the Ritchie stimulator the voltage of the stimulus ranged from 5 to 15 The adjustment of the electrodes on the chain generally produced a response Such a response and that due to the first electrical stimulus might be larger than those subsequently elicited by electrical stimulation

In the experiment of fig 4, a stimulus directly applied to the *longus coll.* muscle at a distance of  $\frac{1}{2}$  cm from the chain did not give rise to P A p effects, although current spread to surrounding tissues was evident This showed that P A p responses we had obtained were not due to stimulation of adjacent structures, and this was confirmed in other experiments

It was pointed out by Daly and Hebb [1942] that pulmonary pressor

The pressor response to MCG stimulation was demonstrated at zero B.P. as had been the response to sympathetic chain stimulation, and similarly could not have been due to a transfer of blood from the

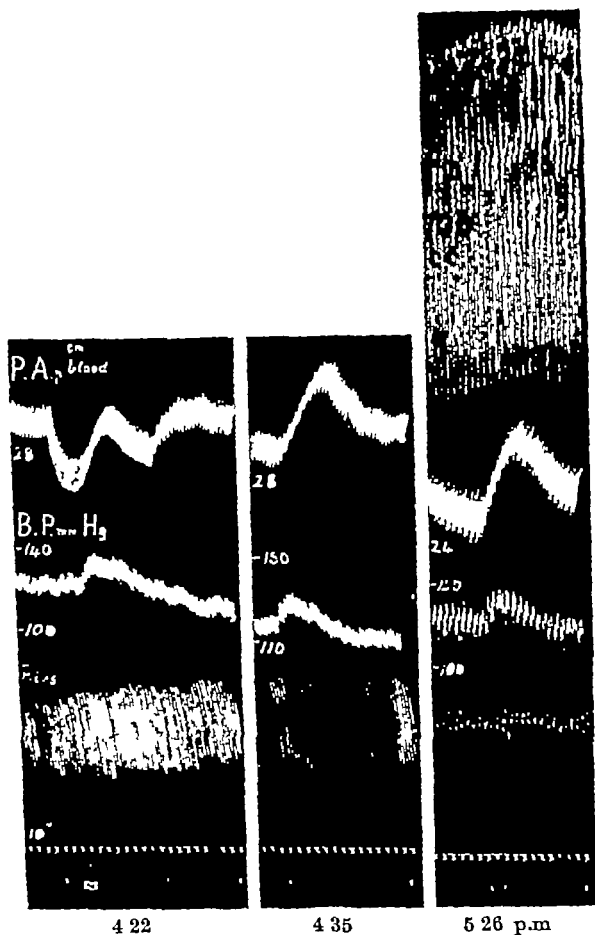


FIG 6—P.L.A. 72 Dog ♀, 6.3 kg, perfused with horse blood at constant blood inflow to both circulations Both CVS cut Duration of perfusion at 4.22 p.m. = 3 hr 32 min

- 4.22 p.m. Positive pressure ventilation stopped at signal marker fall  
Stimulation of L sympathetic chain (c.d. ≈ 7 cm) during solid white signal. Ventilation started at rise of signal marker
- 4.35 p.m. Stimulation of L sympathetic chain (c.d. ≈ 7 cm) during positive pressure ventilation
- 5.26 p.m. Stimulation of L sympathetic chain (c.d. ≈ 7 cm) during negative pressure ventilation

bronchial circulation to the pulmonary circulation (fig 7, b) It was again found that the type of artificial ventilation was not a factor determining the pulmonary vascular responses

Precautions were taken in all experiments to ensure that the temperature of the blood perfusing the animal did not differ significantly from the temperature of the animal itself. Thus the conditions for temperature control were such that neither changes in blood pump output nor redistribution of blood in the animal's tissues, which might take place as a result of physiological responses, would produce a temperature gradient between the blood and the tissues. In fig 5 it will be seen that the temperature of the blood perfusing the systemic circulation (measured in the left auricle reservoir) remained constant. Also the difference between this temperature and that of the P L A preparation (rectal temperature) differed by less than  $0.2^{\circ}\text{C}$ . The difference remained constant during the period when the systemic circulation was switched off, and throughout the series of sympathetic chain stimulations between 3.53 and 5.13 p.m., except during a short interval at 4.56 p.m. The responses obtained are therefore not due to changes in blood temperature. In the same figure it will be noted that 35 seconds after switching off the systemic pump the upper and lower limiting levels of the tidal air (T A) tracing move upwards. The T A tracing is recorded by a spirometer connected to the closed circuit respiratory system into which oxygen is being continuously admitted. The upward movement of the T A tracing therefore denotes a diminished oxygen consumption of the animal after its systemic circulation had been stopped.

Since both vasoconstriction and vasodilatation had been observed in response to stimulation of the sympathetic chain and its ganglia (Table I), it seemed possible that the degree of tone of the pulmonary blood-vessels or their calibre at any given time might have determined the nature of the response. The P A p response might have been influenced by the method of artificial ventilation, because inflation of the lungs by intermittent positive intrapulmonary pressure differs in its effects on the lung blood-vessels from inflation by intermittent extra-pulmonary negative pressure variations. We therefore tested the effects of nerve stimulation when the lungs were under different conditions of mechanical stress. Fig 6 shows that the type of artificial ventilation, or indeed the absence of ventilation, had little effect on the P A p response to sympathetic chain stimulation. The initial fall in P A p in the left-hand tracing is due to the stoppage of positive pressure ventilation, and the final rise to the return of ventilation. These observations were confirmed in a number of other experiments.

*Stellate Ganglion (St G), Ansa subclavia, and Middle Cervical Ganglion (M C G)*—The more usual response to electrical stimulation of the St G and M C G was a P A p rise (fig 3, b) or a decrease both in lung blood inflow and outflow depending upon the method used for assessing changes in calibre of the pulmonary blood-vessels (fig 7, a). Less commonly a diphasic or a weak dilator response occurred (Table I).

## CELL STATIONS

Möllgaard [1910, 1912] showed that the removal of one lung led to degenerative changes in some of the cells of the St G (in cat) and MCG (in dog and cat). Those affected may have been the cells of origin of bronchodilator fibres or of pulmonary vasomotor fibres, but his work clearly indicated the likelihood of our finding pulmonary vasomotor cell stations in one or both of the ganglia. The latter contingency appeared to be the correct one, for the application of

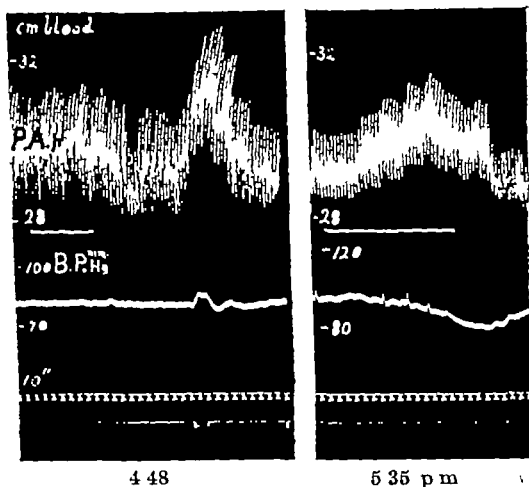


FIG 8—P.L.A. 65 Dog ♀ 8.2 kg, perfused with horse blood at constant blood inflow to both circulations. Positive pressure ventilation. Both CVS cut. Duration of perfusion at 4.48 p.m. = 3 hr 11 min.

4.48 p.m. Nicotine 2 per cent applied to R.M.C.G. (white horizontal line) followed by R.M.C.G. stimulation (c.d. = 8 cm).

5.35 p.m. Nicotine 2 per cent applied to R. stellate ganglion.

nicotine (1 or 2 per cent solution) to either of the two ganglia caused in some experiments an almost immediate rise of P.A.P. and in a lesser number a fall of P.A.P. or a diphasic response (figs 8, 9, and Table II). Further, as will be shown later, stimulation of the sympathetic chain after nicotine application of both ganglia no longer caused pulmonary vasomotor effects, whereas excitation of other nerves which might be regarded as post-ganglionic paths was still effective.

In the few experiments in which a comparison could be made, the direction of the P.A.P. nicotine response with one exception (expt 65) simulated that due to electrical stimulation of the ganglion or of the sympathetic chain (Table II). On two occasions the application of nicotine to the St G caused a P.A.P. rise in steps (fig 8). A possible explanation for this is that successive layers of cells were stimulated as the nicotine diffused into the ganglion.

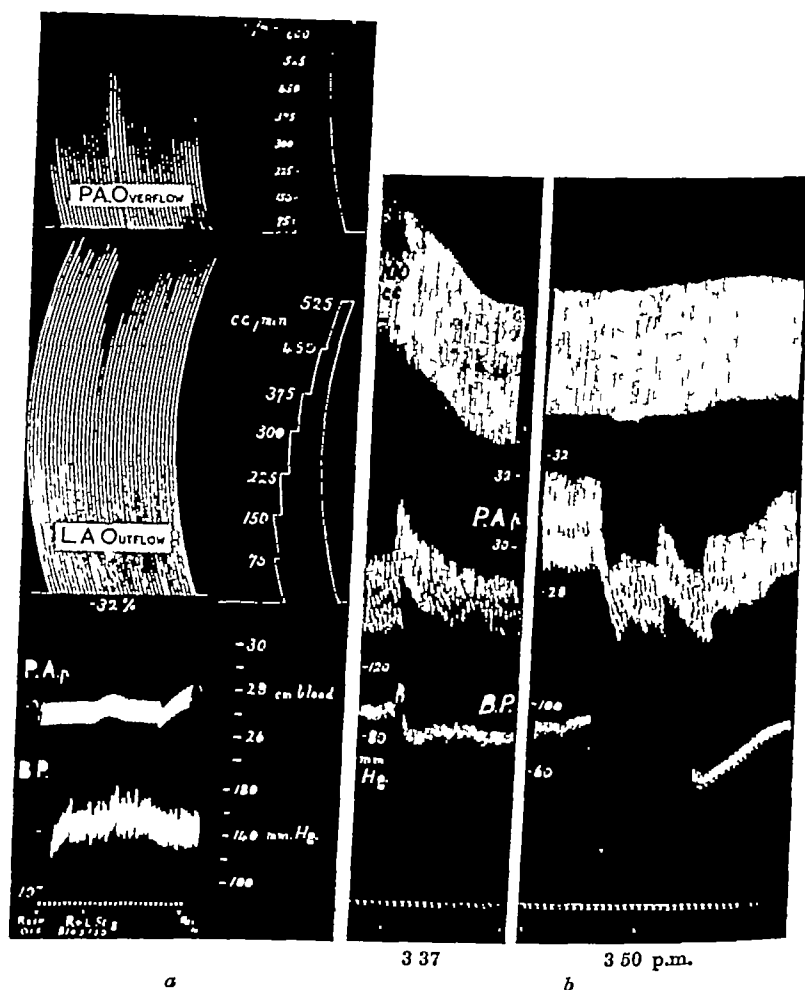


FIG 7—*a* P.L.A. 89 Dog, ♂, 10.0 kg Systemic circulation perfused with "mixed" dog blood at constant blood inflow pulmonary circulation perfused at constant pulmonary arterial pressure R.C.V.S. cut, L.C.V.S. intact Duration of perfusion = 1 hr 6 min Observation made during cessation of artificial ventilation of lungs Stimulation of both stellate ganglia 8/0 5/50

*b* P.L.A. 65 Dog, ♀ 8.2 kg perfused with horse blood at constant blood inflow to both circulations Both CVS cut Duration of perfusion at 3 37 p.m. = 2 hr 15 min

3 37 p.m. Stimulation of R.M.C.G. (c.d. = 8 cm) The tidal air tracing is falling because O<sub>2</sub> admission to the closed air system was temporarily stopped

3 50 p.m. Stimulation of R.M.C.G. (c.d. = 8 cm) during cessation of systemic circulation perfusion

Atropine sulphate 2.0 mg added to the blood at 2 30 p.m.

TABLE II—EFFECT UPON THE PULMONARY ARTERIAL PRESSURE OF ELECTRICAL STIMULATION OF THE SYMPATHETIC CHAIN, THE STELLATE AND MIDDLE CERVICAL GANGLIA, AND OF THE APPLICATION OF 1-2 PER CENT NICOTINE TO THE GANGLIA

Expt	Sympathetic chain, electrical stimulation	Stellate ganglion		Middle cervical ganglion		
		Electrical stimulation	Nicotine	Electrical stimulation	Nicotine	
63				+		Right side
65		+	+	-		Left side
66	+	+	+	+	sl +, -	Right side
67	+		+, -		-	Right side
68	+		sl +		+	Left side
	0				sl +	Right side
73	+		+		+	Left side
	-		-		-	Right side
						Left side

+ =rise of P A p

- =fall of P A p

0 =no effect on P.A p

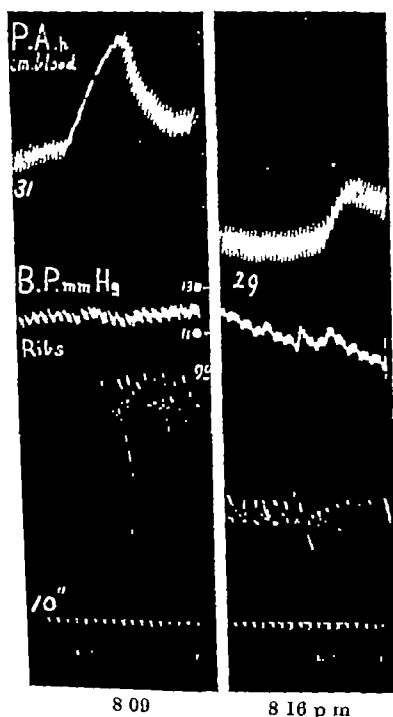


FIG 10—Same experiment as in fig 9 Duration of perfusion at 8 09 p.m. =7 hr 19 min

8 09 p.m Stimulation of L T V S (c d =7 cm )

8 16 p.m Stimulation of ventral limb of L ansa subclavia (c d =7 cm )

The following experiments suggest that the St G and MCG are the main cell stations for the pulmonary vasomotor pre-ganglionic fibres in the upper sympathetic chain, with the possible exception of any which may relay in the SCG or at other points in the trunk of the CVS nerve in which aberrant ganglia may occur. In expt 72 (fig 9) nicotine paralysis of the LMCG alone did not completely block all

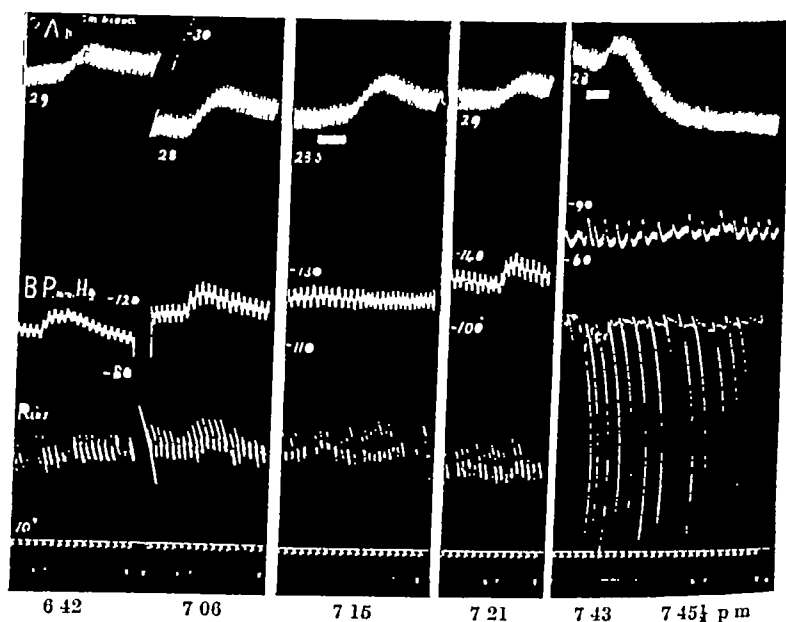


Fig 9—P.L.A. 72 Dog ♀, 6.3 kg, perfused with horse blood at constant blood inflow to both circulations. Both CVS cut. Duration of perfusion at 6:42 p.m. = 5 hr 52 min.

Stimulation of L sympathetic chain (c.d = 7 cm) at 6:42, 7:06, 7:21, and 7:45 1/2 p.m.

7:15 p.m. Nicotine 1 per cent applied to LMCG.

7:43 p.m. Nicotine 1 per cent applied to left inferior cervical ganglion and T<sub>1</sub> ganglion.

the impulses from the L sympathetic chain, but the subsequent application of nicotine to the left inferior cervical ganglion (LICG) and T<sub>1</sub> ganglion, i.e. St G, effectively blocked the remaining impulses. Electrodes were then placed on the caudal end of the cut LTVS and ventral limb of the left *ansa subclavia*. Stimulation in each test caused a rise in PAp (fig 10). The LTVS response may have been due to excitation of post-ganglionic fibres with their cells of origin in the St G or MCG, this will be discussed later. On the other hand, the PAp response to stimulation of the ventral limb of the *ansa* was in all probability due to excitation of post-ganglionic fibres with their cells of origin in the St G. It should be stressed here that this dog was an



## PULMONARY VASODILATATION

Pulmonary vasodilator responses to stimulation of the sympathetic chain and its ganglia were relatively rare (Table I). This militated against a satisfactory examination of the nerve pathways concerned. In experiment 73 evidence was obtained that pulmonary vasodilator fibres were present in the upper thoracic sympathetic chain and that

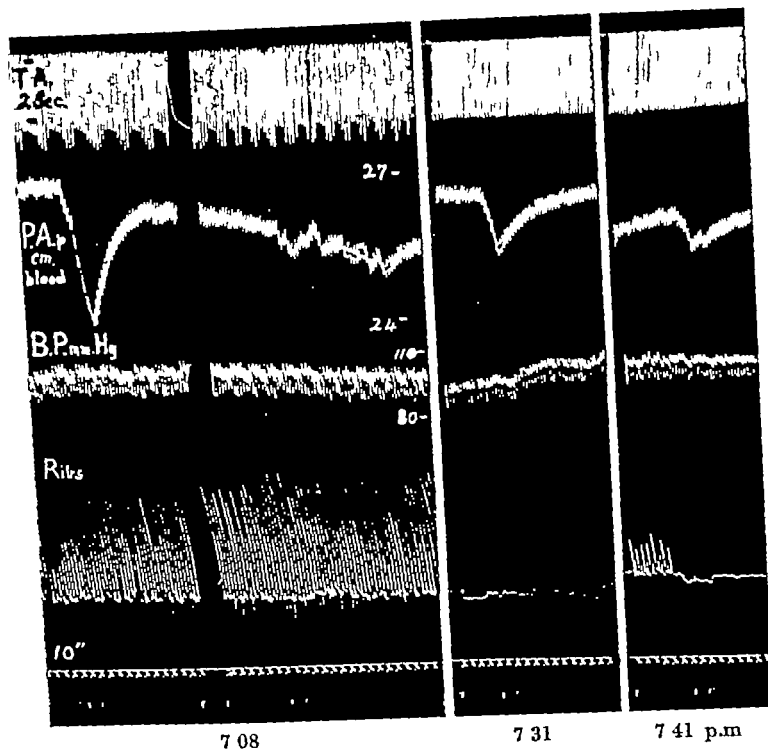


FIG 11B—Same experiment as fig 11A

7 08 p.m. Stimulation of L sympathetic chain followed by stimulation of R sympathetic chain (c d = 3 cm)

7 31 and 7 41 p.m. Stimulation of L sympathetic chain (c d = 3 cm)

Eserine 10 mg added to blood at 7 33 p.m.

their cell stations were probably in the St G and MCG. The experiment was remarkable in that electrical stimulation of the R sympathetic chain and RTVS nerves, as well as stimulation of the R St G and R MCG by nicotine, caused pulmonary vasoconstriction, whereas equivalent stimulations of the L sympathetic chain, L TVS, L St G and L MCG caused pulmonary vasodilatation (figs 11A, B, C).

In fig 11A it will be observed that the second stimulation of the R sympathetic chain failed to elicit a PAp response, although the BP showed a rise of the same order as that produced by the first stimulation

exception to the usual rule that the I C G is fused with the upper three thoracic sympathetic ganglia and sometimes with  $T_4$  ganglion as well. On the contrary, the I C G and  $T_1$  on the left side were represented by discrete ganglia connected together by a loose mesh of nerves. These in turn were connected by fibres 2-3 mm in length to  $T_2$  and  $T_3$  ganglia which were fused together. It was therefore possible in this instance to test the effect of nicotine on each of these ganglia separately. An initial application of nicotine to  $T_2$  and  $T_3$  caused no rise in P.A.p.

Two other experiments in which the order of nicotinising the St G and M C G was reversed gave similar results. In all three experiments the C V S nerves were cut so that any sympathetic pulmonary vaso-motor fibres relaying in the superior cervical ganglion would not come under test.

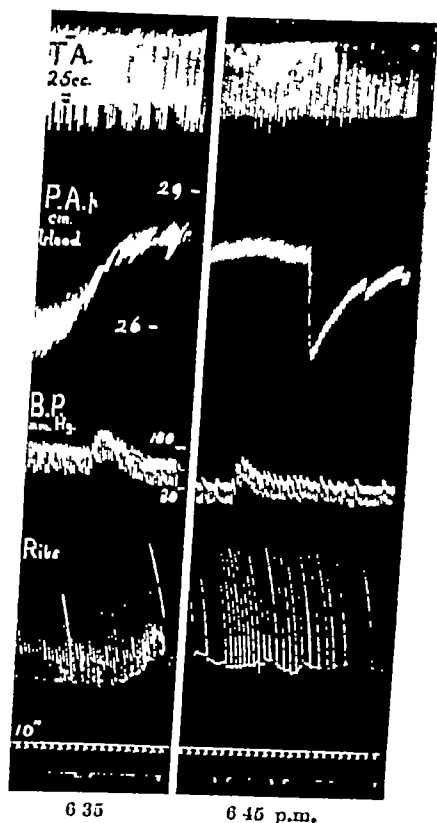


FIG 11A—P.L.A. 73 Dog ♀ 6.0 kg perfused with horse blood at constant blood inflow to both circulations. Both C V S and both recurrent laryngeal nerves cut. Negative pressure ventilation. Duration of perfusion at 6.35 p.m. = 3 hr 41 min.

6.35 p.m. Stimulation of R sympathetic chain (c.d. = 3 cm.)

6.45 p.m. Stimulation of R sympathetic chain (c.d. = 3 cm.) followed by stimulation of L sympathetic chain (c.d. = 3 cm.)

The fact that subsequent stimulation of the R TV S and L TV S nerves gave a pressor and depressor response respectively suggests that they each contained fibres predominantly from the sympathetic chain of the corresponding side

We did not carry out the crucial test of stimulating the L sympathetic chain after paralysis of the ganglia by nicotine. Thus we did not obtain direct evidence that the cell stations for the pulmonary vasodilator fibres reside in the St G and M C G. Even so, if we compare the effects of stimulating the sympathetic chain, the ganglia, and the TV S nerves on each side of the body in experiment 73, it would be remarkable if the St G and M C G were not cell stations for the sympathetic chain pulmonary dilator fibres just as they were found to be for the constrictor fibres. Indeed this is the interpretation we place upon this experiment, so that the results so far obtained may be summarised by the statement that our evidence supports the view that the sympathetic chain contains both pulmonary vaso-constrictor and -dilator fibres both of which relay in the St G and M C G. Their further course is discussed in the next section of this paper.

#### SYMPATHETIC POST-GANGLIONIC FIBRES

These were not fully explored, but electrical stimulation of the ventral limb of the *ansa subclavia* (two experiments) and a branch from the ventral limb of the right *ansa*, which was traced as far as the dorsal surface of the pulmonary artery, all gave a rise of P A p. The probability that these fibres were of vagal origin is remote, and therefore they may be reasonably regarded as belonging to the thoracic sympathetic outflow.

Stimulation of the TV S nerves almost invariably produced changes in P A p. A pressor response predominated (Table I, and figs 10, 11c, 12, 13), and persisted after the intravenous injection of nicotine (10-20 mg), which was sufficient to block pre-ganglionic impulses. Excitation of branches of the TV S, namely the left ventrolateral cardiac nerve and a looping branch of the R TV S nerve (fig 1, a), also caused a rise of P A p.

We have demonstrated that after the application of nicotine to the St G and M C G the pulmonary pressor response to stimulation of the sympathetic chain disappears, whereas that to TV S stimulation persists (figs 9, 10). These observations are not unequivocal evidence that the vasoconstrictor fibres in the TV S nerves have their cell stations in the St G and M C G. They might be derived from other sources. We therefore tested the effect of stimulating the sympathetic chain before, during, and after cooling the TV S nerve, and found that the P A p rise due to sympathetic chain stimulation was significantly reduced by cooling the TV S nerve of the same side (fig 12).

A third stimulation of the R sympathetic chain merely produced irregular oscillations of the P A p record (fig 11B, 7 08 p m ) We are at a loss to account for this phenomenon, we have, however, observed it on other occasions Examination of the R sympathetic chain just after the second stimulation showed some drying, but if this was the cause of loss of activity of the pulmonary vasomotor fibres it apparently

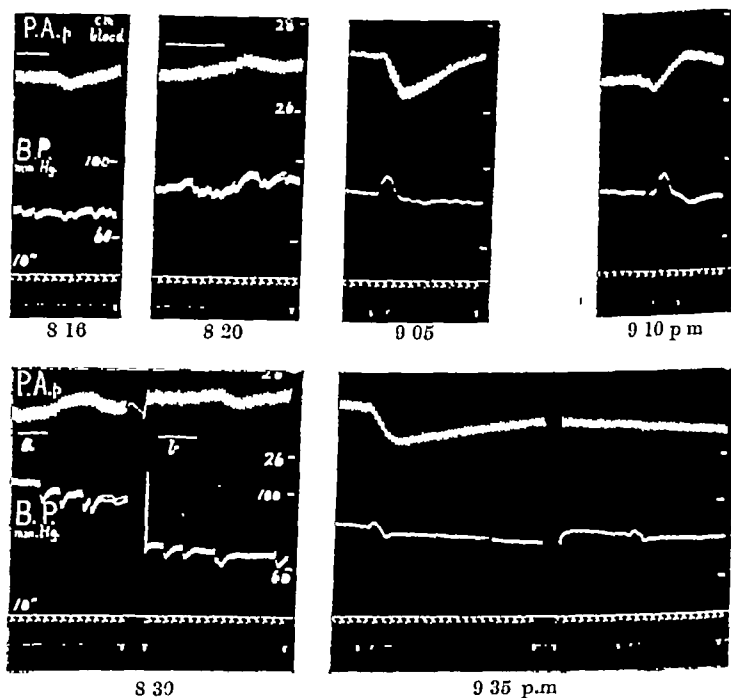


FIG 11c—Same experiment as in fig 11b

- 8 16 p.m Nicotine (1 per cent) on L St G  
 8 20 p.m Nicotine on R St G  
 8 30 p.m Nicotine on R MCG (a) Nicotine on L MCG (b)  
 9 05 p.m LTVS stim c d = 3 cm  
 9 10 p.m RTVS stim c d = 3 cm  
 9 25 p.m Ergotoxine ethanesulphonate 30 mg added to blood  
 9 35 p.m 1st stimulus on LTVS (c d = 3 cm) 2nd stimulus on RTVS (c d = 3 cm)

had not affected the vasoconstrictor fibres to the systemic circulation (see fig 11A) Figs 11A and 11B show that the vasodilator response to L sympathetic chain excitation diminished with successive stimulation but was not enhanced by a small dose (1 mg) of eserine The preparation was in poor condition at this stage and respiratory activity had ceased In spite of this, small but definite P A p responses were obtained by painting the St G and MCG with nicotine, the response being in each case pressor to stimulation of the ganglia on the right side and depressor to stimulation of the left ganglia (fig 11c, 8 16–8 30 p m)

## NATURE OF PULMONARY SYMPATHETIC VASOMOTOR FIBRES

It has been repeatedly demonstrated that the pulmonary vasoconstriction following stimulation of the chain, the St G, the MCG, and the TVS nerves (*a*) is not enhanced by eserine (*c* 1 in  $10^6$ ), (*b*) occurs in atropinised preparations (*c* 1 in 250,000), and (*c*) is invariably

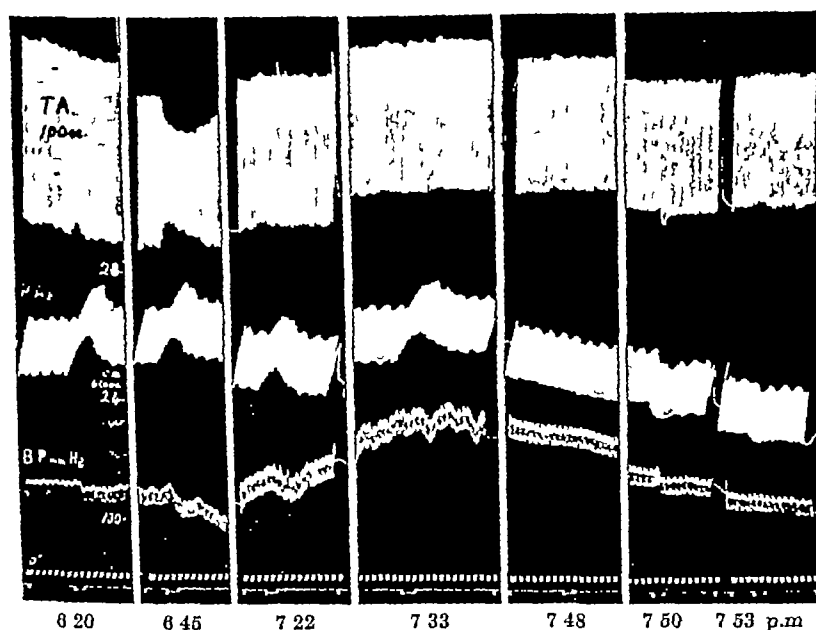


FIG 13—P.L.A 83 Dog, ♂, 6.8 kg, perfused with horse blood at constant blood inflow to both circulations. Negative pressure ventilation. Both CVS cut. Duration of perfusion at 6.20 p.m. = 3 hr 53 min.

- 6.20 p.m. LTVS stimulation, 8/10/50
- 6.28 p.m. Eserine 0.5 mg
- 6.45 p.m. LTVS stimulation, 8/10/50
- 6.51 p.m. Atropine sulphate 5.0 mg
- 7.22 p.m. LTVS stimulation, 10/10/50
- 7.33 p.m. R sympathetic chain stimulation, 4/10/50
- 7.38 p.m. Ergotoxine ethanesulphonate 4 mg
- 7.48 p.m. R sympathetic chain stimulation, 4/10/50
- 7.50 p.m. LTVS stimulation, 10/10/50, showing current spread.
- 7.53 p.m. LTVS stimulation, 8/10/50

either suppressed, partially or completely, or reversed by ergotoxine ethanesulphonate (*c* 1 in  $10^6$  to 1 in 500,000) (figs 11c, 13). These observations are compatible with the view that the vasoconstrictor fibres are adrenergic. Daly and Euler [1932] had previously reported that ergotamine tartarate suppressed the TVS vasoconstrictor response. The effect of cocaine on the response to nerve stimulation has not been tested.

Analysis of the dilator responses presented more difficulty. In only

Total suppression of the P A p response while the main trunk of the T V S nerve was cooled would not be expected, because branches of the T V S which came off above the level of cooling also carry pulmonary vasoconstrictor fibres. There is therefore no reason to doubt that some of the pulmonary vasomotor fibres found in the T V S are the post-ganglionic continuation of pre-ganglionic fibres issuing from the cord

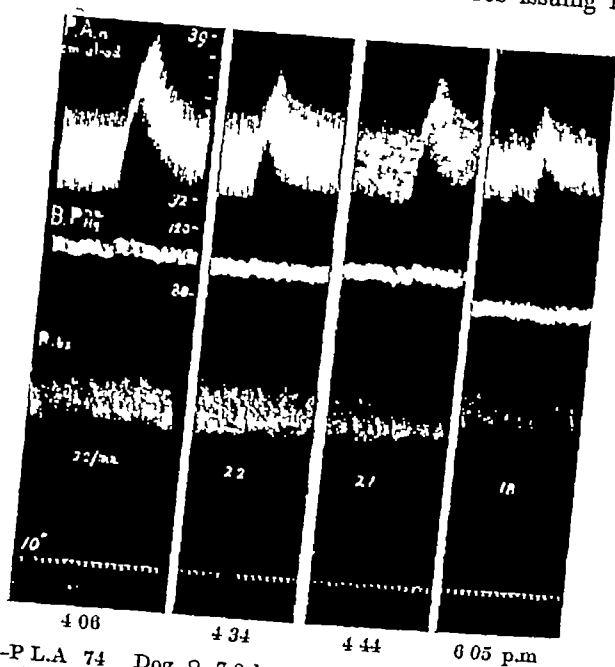


FIG 12—P L A 74 Dog ♀, 7.8 kg, perfused with horse blood at constant blood inflow to both circulations. Positive pressure ventilation. Both C V S and both recurrent laryngeal nerves cut. Duration of perfusion at 4.00 p.m. = 1 hr 6 min. R sympathetic chain stimulated (c.d. = 11 cm) at 4.00, 4.34 and 4.44 p.m. The R T V S nerve was cooled at superior azygos vein level between 4.15 and 4.30 p.m. Atropine sulphate (4.0 mg) added to blood at 2.55 p.m. 6.05 p.m. = two successive stimulations of the R T V S nerve, 1st, c.d. = 11 cm, 2nd c.d. = 3 cm.

in the upper thoracic sympathetic outflow and relaying in either the St G or M C G.

We have less information concerning the path by which sympathetic vasodilator fibres reach the lungs. Although the chain, the St G, the M C G, and the T V S nerves contain pulmonary vasodilator fibres (see expt 73, and figs 11A, B, C), we lack evidence that these fibres are a direct route to the lungs. There was no opportunity for testing whether cooling the T V S nerve diminished the dilator response to sympathetic chain stimulation, or whether the T V S dilator response was retained after intravenous injection of nicotine.

T V S stimulation suggests that the preparation was still fully atropinised although the atropine had been added to the blood more than four hours before. When eserine was given, stimulation of the T V S was not followed by bronchoconstriction. Therefore atropine in large enough doses to paralyse the bronchoconstrictor fibres apparently fails

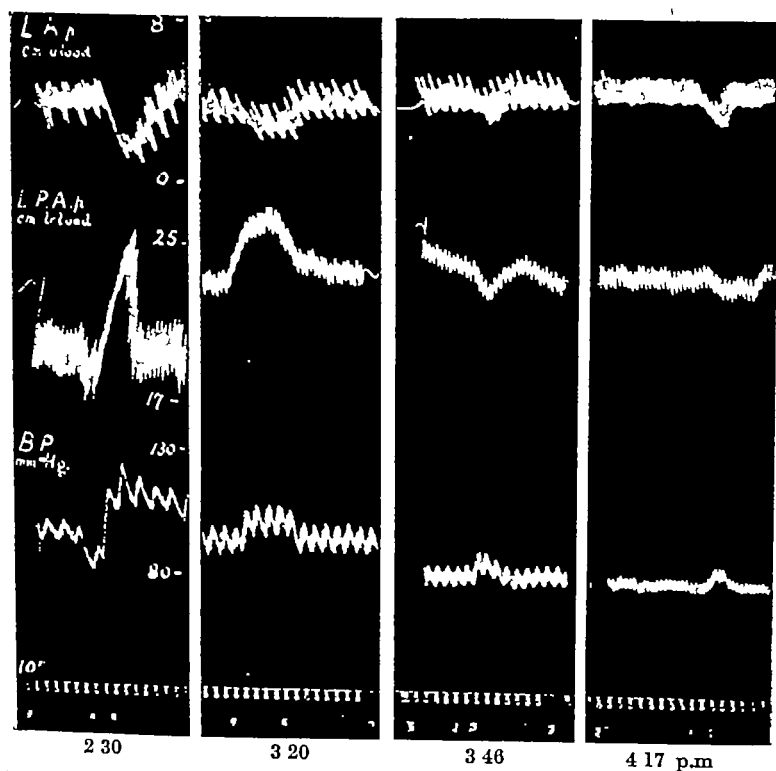


Fig 15—Left lung perfusion *circa* 480 c.c./min. Dog, ♀ 80 kg. Morphine, chloralose (Method of Daly and Duke, 1948)

- 1 48 p.m. Atropine sulph. 20 mg
- 2 30 a.m. Stimulation L sympathetic chain, 20/10/50
- 3 20 p.m. Stimulation L sympathetic chain, 10/100/5
- 3 22 p.m. Ergotoxine ethanesulphonate 10 mg
- 3 46 p.m. Stimulation L sympathetic chain, 10/100/5
- 3 50 p.m. Atropine sulph. 20 mg
- 4 17 p.m. Stimulation L sympathetic chain, 10/100/5
- L A p = left auricle pressure
- L P A p = left pulmonary arterial perfusion pressure

to paralyse pulmonary fibres which are responsible for the vasodilator response after the administration of ergotoxine. We also carried out experiments to ensure that the preparations were fully atropinised. In these experiments atropine given after ergotoxine sometimes reduced but never abolished the P A p depressor response to stimulation of the sympathetic chain and T V S nerves (fig 15)

one experiment were we able to observe the effect of eserine on an *initial* dilator response to sympathetic chain stimulation in a non atropinised preparation in which bronchoconstrictor responses were absent (fig 11B) In this experiment eserine (concentration in blood 1 in  $2 \times 10^6$ ) had no potentiating effect

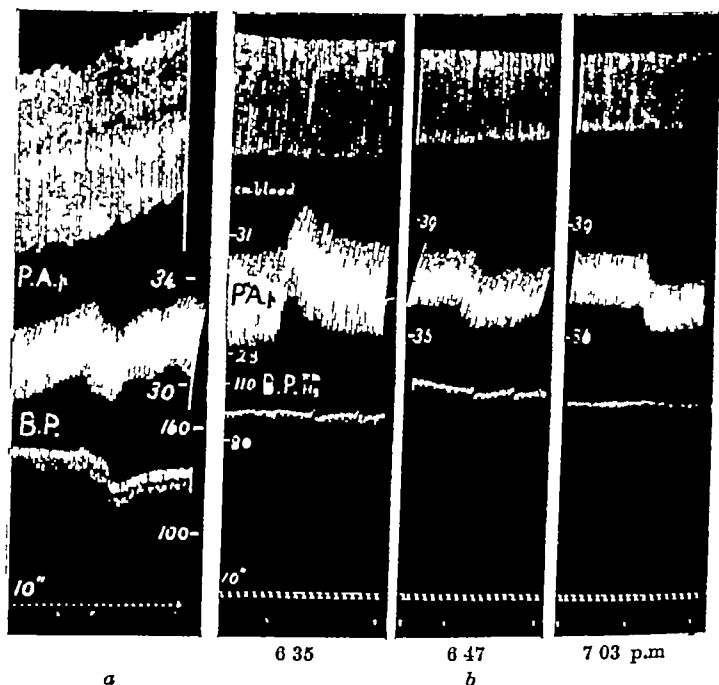


FIG 14 a—Same experiment as illustrated in fig 3, b, which shows response before ergotoxine

Stimulation of R St G 15/10/50, 15 min after the addition to the blood of ergotoxine ethanesulphonate 5.0 mg  
Atropine sulphate 2.0 mg added to blood 155 min previously

FIG 14 b—Same experiment as in fig 7, b  
6 35 p.m Stimulation of R T V S (c d = 0 cm)  
6 37 p.m Ergotoxine ethanesulphonate 4.0 mg  
6 47 p.m Stimulation of R T V S (c d = 0 cm)  
6 50 p.m Eserine 3.0 mg  
7 03 p.m Stimulation of R T V S (c d = 0 cm)  
Atropine sulphate 2.0 mg added to the blood at 2 30 p m

The other type of vasodilator response we had the opportunity of examining was that present after the ergotoxine reversal of the usual pressor effects obtained by stimulation of the sympathetic chain, St G and T V S nerves. This type of pulmonary vasodilatation occurred in atropinised preparations (fig 14). In the experiment illustrated in fig 14, b, the absence of a significant bronchoconstrictor response to



Pulmonary vasodilator fibres have not been so fully explored because a dilator response to sympathetic chain stimulation is a rare phenomenon. The observations in expt 73, in which electrical stimulation of the left sympathetic chain and of the LTVS nerve, and nicotineisation of the LStG and LMCG, all produced pulmonary vasodilatation, can be explained on the grounds that these ganglia and the TVS form one of the pathways to the lungs for pulmonary dilator fibres from the sympathetic chain. The evidence for this opinion gains strength from the parallel constrictor responses obtained on the other side of the body in the same experiment but is less secure than that produced for vasoconstrictor fibres. The effect of cooling the TVS nerve on the dilator response to sympathetic chain stimulation was not carried out, nor were tests made to determine whether nicotine paralysis of the ganglia would prevent the dilator response to sympathetic chain stimulation.

In the light of the critical analyses by McSwiney and Spurrell [1933] and Babkin [1944], which failed to establish the claims of Kiss [1931, 1932] that some efferent vagal fibres belong to the sympathetic system, we regard it as unlikely that the sympathetic pulmonary vasomotor fibres are derived from sources other than the thoracic sympathetic chain. The presence of pulmonary vasomotor fibres in the cervical vagosympathetic nerves in no way affects this belief, for they might well be derived from the thoracic sympathetic with their cell stations in the superior cervical ganglion.

The use of drugs as diagnostic agents has led to results which not only strengthen our opinion that the sympathetic chain ganglia and TVS nerves are indeed one of the sympathetic vasomotor paths to the lungs, but also shed some light on the nature of the fibres concerned. All the evidence we have obtained is compatible with the view that the pulmonary vasoconstrictor fibres are adrenergic, because pulmonary vasoconstrictor responses to nerve stimulation occur in atropinised preparations, they are not enhanced by eserine and they are suppressed or reversed by ergotoxine. Pure dilator responses have also been obtained on stimulating the same path, but we have not got enough evidence to be certain of the nature of the nerve-fibres concerned.

The absence of any potentiating effect of eserine ( $1 \text{ in } 2 \times 10^6$ ) on the vasodilator response to sympathetic chain stimulation in a single experiment, and the fact that atropine fails to suppress the post-ergotoxine depressor response both to sympathetic chain and to TVS stimulation, suggest that some of the pulmonary dilator fibres may be adrenergic. We have not, however, ruled out the possibility that they may be atropine resistant fibres, either cholinergic or of unknown nature.

The presence of vasomotor fibres to the lungs other than adrenergic or cholinergic was suggested by Ungar, Grossiord, and Brincourt [1936]. They worked on atropinised dogs, and their results led them to conclude

The persistence of a depressor response after ergotoxine and atropine is evidence of adrenergic pulmonary vasodilator fibres. It is possible however, that they are atropine-resistant cholinergic fibres. It should be noted that pulmonary vasodilator fibres which may be demonstrated on occasion in the C V S are readily suppressed by atropine in doses similar to those used in the present experiments [Daly, Duke, and Hebb, 1948]

A final interpretation cannot be placed upon these results until quantitative studies of the phenomena have been carried out. Studies of the fibres in the T V S nerves will prevent considerable experimental difficulties because they should include tests to determine the action of eserine on the post-ergotoxine pulmonary depressor response. Eserine, however, causes bronchoconstriction with attendant passive effects on the pulmonary vascular bed, and this complicates the interpretation of P A p responses.

#### DISCUSSION AND CONCLUSIONS

Under experimental conditions which rule out the participation of current spread, of blood temperature changes and of passive changes in the pulmonary vascular bed due to cardiac or bronchomotor events, it has been found that electrical stimulation of the upper thoracic sympathetic chain more often produces pulmonary vasoconstriction than pulmonary vasodilatation. The vasoconstrictor response is present when the systemic arterial pressure is zero, which indicates that an alteration in the transfer of blood from the bronchial to the pulmonary circulation is not the cause of the observed pulmonary arterial pressure change.

Stimulation of the St G, M C G, or T V S nerves under similar experimental conditions also produces pulmonary vasomotor responses, and again vasoconstriction is the more usual effect.

Pulmonary vasoconstrictor fibres derived from the sympathetic chain have been shown to relay in both the St G and the M C G. The post-ganglionic fibres run in the T V S nerves and their branches. Our evidence for this is

- (1) The initial application of nicotine to one or both ganglia causes pulmonary vasoconstriction
- (2) The pulmonary pressor response to stimulation of the sympathetic chain is not abolished until both ganglia have been paralysed by nicotine
- (3) After both ganglia have been paralysed by nicotine, stimulation of the T V S nerves still causes an increase of P A p
- (4) Cooling the main trunk of the T V S nerve reduces the P A p response to stimulation of the sympathetic chain

experiments the results obtained are representative of those obtained in animals with an intact circulation. It might be added that Dirken and Heemstra [1948], working on anaesthetised rabbits, found that resection of the sympathetic chain on one side increased the oxygen uptake of the homolateral lung, and stimulation of the upper thoracic ganglion caused in most cases a diminution in oxygen uptake of the homolateral lung. They do not, however, regard these results as unequivocal evidence of a sympathetic pulmonary vasoconstrictor mechanism.

Reviewing all the evidence obtained from animals under a variety of experimental conditions, there seems to be no doubt that a sympathetic pulmonary vasoconstriction is more readily demonstrated than a vasodilatation. This finding does not justify the assumption that the influence of the sympathetic pulmonary vasomotor nerves in the normal animal is predominantly vasoconstrictor, although we regard it as indicating that potentially the sympathetic nerves can produce pulmonary vaso-constriction or -dilatation in the normal animal.

We have been unable to discover why a sympathetic pulmonary vaso-constrictor response occurs in some experiments and a -dilator response in others. As previously mentioned, the specific nature of the perfusate does not appear to be a dominant factor. The anaesthetic is a complication common to all the experiments which have been quoted. In our own experiments chloralose was used, although we have obtained similar results using Pernocton as an anaesthetic. In this connexion Neil, Redwood, and Schweitzer [1948] found that intravenous injection of chloralose in the decerebrate cat, or in cats under nembutal anaesthesia, converted the depressor response to electrical stimulation of the sinus nerve to a marked pressor response. But in the experiments of others in which sympathetic pulmonary vasoconstriction was obtained, ether, chloroform, and curare were used, so the predominant pressor response does not appear to be dependent upon the use of a specific anaesthetic. It occurred to us that the anaesthetic, by causing marked dilatation of the pulmonary vessels, might account for our failure to demonstrate sympathetic vasodilatation more frequently. But no evidence has been obtained that this is so, on the contrary, the pulmonary vessels appeared to be constricted rather than dilated. In the vast majority of experiments the lungs remained somewhat pale in colour, and the blood-flow through the lungs was less than that through the systemic circulation when the pulmonary arterial and systemic arterial pressures were kept within normal physiological limits, which suggests that loss of tone of the pulmonary blood-vessel was not the cause of the low proportion of sympathetic vasodilator responses.

Without further evidence the significance of a predominant pulmonary vasoconstrictor response to sympathetic stimulation cannot be ascertained, but if eventually it is found to be absent in normal

that the phrenic nerves contain histaminergic pulmonary vasodilator fibres. These were regarded as antidromic fibres with their cells of origin in the posterior root ganglion of  $C_4$ - $C_7$ , the axons of which passed down the phrenic nerves to join the stellate ganglion and upper thoracic sympathetic chain, thence to the lungs. The existence of such fibres requires confirmation, indeed later work by Ungar and Parrot [1938, 1939] led them to believe that antidromic vasodilatation is due to the release of a substance which bears a closer resemblance to adrenoxime than to histamine. Since the pulmonary vasodilator fibres which we describe appear to have their cell stations in the St G or MCG, they are not antidromic.

In our present series of experiments we have found that stimulation of pulmonary vasoconstrictor fibres in preparations perfused at constant blood inflow to the P A produces an increase of P A p of 10-15 per cent of its initial value, which represents a pressure change of less than 5 mm Hg. This observation by itself might suggest that the functional activity of the pulmonary nerves is weak. However, in preparations perfused at constant P A p, stimulations of the same magnitude produce approximately a 30 per cent decrease in the total volume of blood flowing through the pulmonary circulation, which might well reflect events of considerable physiological significance. Our experiments do not fully explain these events, but it is evident that the small changes in P A p which occur under a particular set of experimental conditions may give a false impression of the order of vascular events which actually take place.

How far may the types of pulmonary vascular responses to nerve stimulation which we have described be regarded as those which are potentially operative in the normal animal? It might be argued that the introduction of heparinised shed blood, either homologous or heterologous, into an anaesthetised animal which has already suffered a considerable degree of surgical shock would give rise to a series of complex biological events, both humoral and nervous, inevitably leading to an abnormal physiological state of the pulmonary vascular bed. Thus the responses of the pulmonary blood-vessels to pulmonary sympathetic nerve stimulation under the conditions we have imposed would not necessarily bear any relation to those occurring in the normal animal. This cannot be denied, yet it is remarkable that a sympathetic pulmonary pressor response is predominant in isolated perfused lungs, in the perfused living animal, in the perfused left-lung animal, and in the anaesthetised animal with intact circulation (for literature see the opening paragraph of this paper). Further, it has been found in this laboratory that this same type of response is predominant in the perfused living animal (dog) irrespective of whether the perfusate is the blood of dog (heparinised or defibrinated), horse, or sheep. We may take it, therefore, that in spite of the artificial conditions of perfusion in our

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unanæsthetised animals, its presence under experimental conditions must be due to circumstances common not only to P.L.A. preparations but to all experiments so far reported

Finally, mention should be made of one curious feature of the results, namely, the limitation of pure pulmonary vasodilator responses to stimulation of nerves on the left side of the body (Table I). Diphasic responses occurred equally to stimulation of the nerves on each side of the body. The predominance of dilator responses to stimulation of the nerves on the left side is statistically significant ( $\chi^2$  test,  $P < 0.1$ ). We have no grounds for the belief that this result has its origin in an experimental error, and the only reasonable explanation we have to offer is that it is due, during ontogenesis, to the development of an unequal distribution of pulmonary vasoconstrictor and -dilator fibres to each side of the body.

#### SUMMARY

In the perfused living animal, under conditions which eliminate cardiac and bronchomotor effects, stimulation of the upper thoracic sympathetic chain, the stellate ganglion, the middle cervical ganglion, and the thoracic vagosympathetic nerves causes a rise in pulmonary arterial pressure of 10–15 per cent at constant pulmonary blood inflow. More rarely a fall in pulmonary arterial pressure takes place. Stimulation of these same nerve structures at constant pulmonary arterial pressure perfusion may cause a diminution in lung blood-flow by as much as 30 per cent.

The rise in pulmonary arterial pressure is not due to changes in the amount of blood transferred from the bronchial to the pulmonary vascular bed, nor is it dependent upon the method used for artificial ventilation of the lungs.

Results are described which are consistent with the view that the upper thoracic chain, stellate ganglion or middle cervical ganglion, and thoracic vagosympathetic nerves constitute a pre-ganglionic-ganglionic-post-ganglionic path for adrenergic pulmonary vasoconstrictor fibres. The same path probably carries pulmonary vasodilator fibres, the nature of which is discussed.

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REFLEX REBOUND IN EXTENSOR MUSCLES By J DEL  
CASTILLO-NICOLAU and A SCHWEITZER From the Department  
of Physiology, University College, London, W C 1

*(Received for publication 19th November 1948)*

It is well known that inhibition of a reflex is often followed by a marked increase in its activity. For instance, mechanical stimulation of the hamstring muscles inhibits the knee jerk, which subsequently shows a phase of hyperexcitability, inhibition of rhythmic stepping movements is followed by a period of increased excitability, inhibition of extensor reflexes during reflex activation of ipsilateral flexor muscles is followed by increased extensor reflex excitability, as shown by the potentiation of the extensor thrust.

Rebound contraction was regarded as a special case of "successive spinal induction" [Sherrington, 1905, 1906]. It was thought that the hyperexcitability following reflex inhibition was such that the cessation of the inhibitory stimulus alone would suffice to produce a reappearance of the antagonistic reflex previously depressed. Rebound contraction following inhibition of extensor muscles was held to be inherent in the nature of the flexor reflex ("diphasic reflexes"). Rebound phenomena were explained on the basis of two hypotheses: (1) a change, of origin unknown, from a central inhibitory to a central excitatory state, (2) the presence in afferent nerves of fibres with excitatory and inhibitory central connections, during stimulation the reflex inhibitory effect would be predominant, but, in virtue of a prolonged after-discharge, the excitatory connections would become operative on discontinuing the stimulation and cause rebound contraction [Sherrington and Sowton, 1911a].

This hypothesis of the essentially central origin of the rebound phenomenon received support from Graham Brown [1911], Forbes [1921], Forbes, Davis and Lambert [1930], and Creed, Denny-Brown, Eccles, Liddell and Sherrington [1932]. It was based on Sherrington's

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As Sherrington and Sowton [1911 *b*] have shown, reflex responses depend on the strength of stimulation. Thus weak stimuli may produce ipsilateral contraction of the vasto-crureus, while strong stimulation in the same preparation may produce extensor relaxation. Our observations also confirm Sherrington's [1908] statement that the rebound phenomenon is difficult, if not impossible, to elicit if the animal is asphyxiated or under the influence of anaesthetics.

Three types of rebound contractions of the vasto-crureus muscle were studied

- (a) Rebound contraction following reflex inhibition
- (b) Rebound contraction after reflex contraction caused by ipsilateral stimulation
- (c) Rebound contraction after reflex contraction caused by contralateral stimulation

(a) *Rebound Contraction following Reflex Inhibition*

Our observations completely agree with Sherrington's [1908, 1909] analysis of this phenomenon. There was no correlation between the

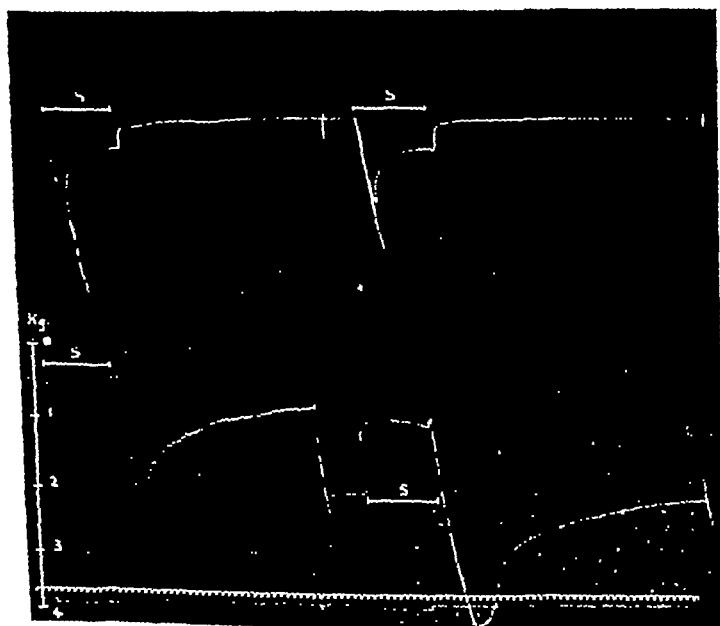


FIG 1—Decerebrate cat. Stimulation of left popliteal nerve (central end) with 70 cys/sec, 100 m sec pulse duration and 25 v. Records from above downwards: contractions of hamstring muscles, contractions of quadriceps, time in sec, signal. Horizontal bars marked S indicate period of stimulation. Note increase in rebound contraction of quadriceps with increased initial stretch of muscle.

[1909] observations of the persistence of rebound after deafferentation of the muscle [also Graham Brown, 1911]

The number of phenomena included in the term "rebound" increased, so that for Graham Brown [1911] rebound "was any movement either of relaxation or of contraction which succeeds the reflex act on the termination of its stimulus". In this paper the term will be used in a more restricted sense to denote reflex contractions appearing immediately after cessation of a stimulus which elicited a reflex contraction or inhibition, the tension developed by the rebound contraction being greater than that present in the pre-stimulatory period.

The purpose of this investigation was to re-examine the rôle in the mechanism of the rebound phenomenon of afferent impulses originating from the vasto-crureus muscle after reflex contraction or inhibition.

### METHODS

Cats were decerebrated under light ether anaesthesia. Drills were inserted into the femur for rigid limb fixation. The central end of the popliteal nerve was stimulated by means of a Collison fluid electrode, using a square wave stimulator with independent frequency of stimuli, pulse duration and voltage output control. The peroneal nerve was sectioned. Lumbar laminectomy was performed, preparatory to transection of spinal posterior roots. The patellar tendon was freed from its attachments and connected to the spring recorder of a Brown Schuster myograph. Frequently the contractions of the hamstring muscles were recorded by fixation of the tibia to a second lever on the myograph after amputation of the foot.

In all records, downward movements of the recording systems correspond to contractions of the muscles concerned. In all experiments where posterior spinal roots had been sectioned, the exact position of the roots affected was verified post mortem.

### RESULTS

Centripetal afferent nerve stimulation produced striking variations in the reflex responses which followed. Ipsilateral reflex contraction of the flexor and ipsilateral relaxation of the extensor muscles, the classical response, was most frequently observed. However, flexor contraction was often accompanied by reflex contraction of the ipsilateral extensor muscles, a response which Graham Brown [1911] called the "dilemma of reaction". Sometimes, in addition to post stimulatory contractions of the muscles under observation, the stimulation caused generalized movements of the musculature of the whole animal, which persisted after cessation of the stimulus. These generalized contractions would be regarded by Graham Brown [1911] as manifestations of rebound.



rebound appeared in the form of rhythmical contractions maintained for periods up to 30 seconds. As in the condition of rebound contraction following reflex inhibition, its strength after reflex contraction depended on the original degree of stretch to which the muscle was subjected (fig 2)

(c) *Rebound Contraction after Reflex Contraction caused by Contralateral Stimulation*

Rebound contraction of the quadriceps muscle was often recorded after cessation of contralateral afferent stimulation which had caused a crossed extensor reflex. This type of rebound contraction was often very powerful, and occurred after the muscle had returned to its pre-stimulatory base-line. It showed a considerable degree of variability on frequent repetition of the contralateral sensory stimulation in the same animal. On the whole, the strength of the rebound appeared to be related to the strength of stimulation, although, in some cases, this correlation could not be demonstrated. It was not possible to demonstrate any relationship between the strength of rebound contraction and the degree of stretch previously applied to the muscle.

EFFECT OF DEAFFERENTATION OF THE MUSCLE ON THE REBOUND CONTRACTION

(a) *Rebound Contraction following Reflex Inhibition*

Transection of the sixth posterior lumbar root usually completely abolished the rebound contraction which previously followed reflex inhibition. In fact, this procedure altered the character of the response to ipsilateral sensory stimulation. Reflex inhibition was no longer obtained and reflex contraction occurred, which disappeared soon after cessation of the stimulation. There was no rebound contraction (fig 3).

In a few instances, transection of the sixth posterior lumbar root did not completely abolish rebound. When, in addition, the seventh posterior lumbar root was cut in these cases, rebound contraction was finally abolished.

In experiments where the seventh posterior lumbar root alone was transected, rebound contraction was often decreased and inconstant in appearance.

In some cases, transection of the fifth, sixth and eighth lumbar and all sacral posterior roots, the seventh lumbar root remaining intact, did not abolish rebound contraction, although it appeared to be more variable in extent. Frequently the character of the reflex response preceding rebound was changed, reflex inhibition occurring where, prior to sectioning of these posterior roots, ipsilateral sensory stimulation had

actual mechanical lengthening of the muscle occurring during the phase of reflex inhibition and the extent of the consequent rebound contraction. In fact, in many instances powerful rebound contraction occurred in the absence of any detectable preceding lengthening of the muscle.

It should be added, however, that in our experience the strength of rebound contraction appeared to be related to the degree of stretch to which the muscle was subjected prior to reflex inhibition. Fig 1 clearly shows the increase in rebound strength with an increase in the stretching force previously applied to the muscle.

(b) *Rebound Contraction after Reflex Contraction caused by Ipsilateral Stimulation*

In animals with a low degree of decerebrate rigidity ipsilateral centripetal stimulation usually caused reflex contraction of the quadriceps muscle. This contraction ceased after discontinuing the stimulation and was not followed by rebound.

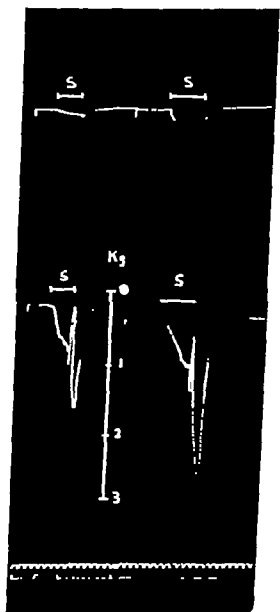


FIG 2—Records as in fig 1. Rebound contraction following quadriceps contraction after ipsilateral sensory stimulation (reaction of dilemma<sup>1</sup>). Rebound contraction increased after increasing initial stretch of muscle.

With marked decerebrate rigidity of the vasto-crureus, ipsilateral sensory stimulation usually produced an inhibitory response, but it frequently caused reflex contraction, and when this occurred the cessation of stimulation was followed by marked rebound contraction. Often this

produced excitation (fig 4, A and B) This figure also shows a distinct alteration in the appearance of the flexor reflex after transection of these posterior nerve-roots, no doubt due to the reduction in the number of afferent fibres reaching the spinal cord subserving the flexor reflex

(b) *Rebound Contraction after Reflex Contraction caused by Ipsilateral Stimulation*

Transection of the sixth posterior lumbar root usually completely abolished this type of rebound contraction (fig 5) In rare instances,

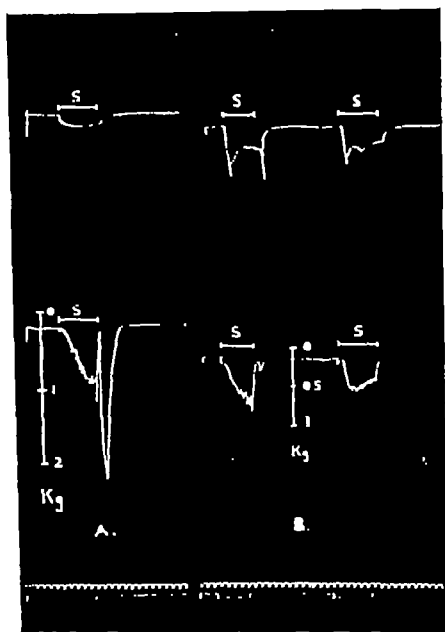


FIG 5—Records as in fig 1 “Reaction of dilemma” Between Sections A and B division of the sixth lumbar posterior root Dilemma reaction persists, but rebound contraction abolished.

feeble and inconstant rebound contractions were still present after this procedure, sectioning of the seventh posterior lumbar root would then finally abolish the rebound

(c) *Rebound Contraction after Reflex Contraction caused by Contralateral Stimulation*

Transection of the sixth posterior lumbar root completely, or almost completely, abolished rebound contraction following a crossed extensor contraction In no instance was rebound observed after sectioning of

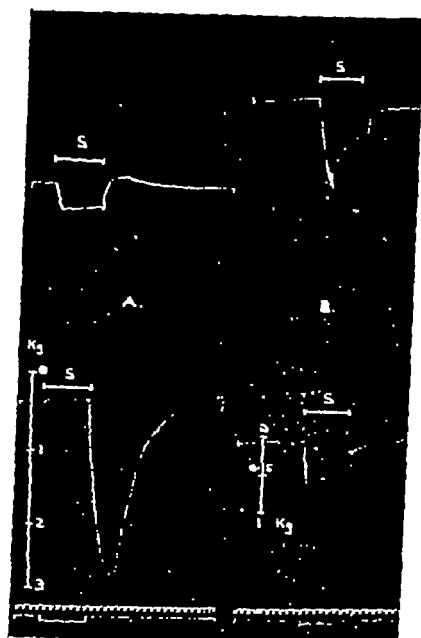


FIG 3—Records as in fig 1 Between sections A and B division of sixth lumbar posterior root. Note absence of rebound contraction following ipsilateral sensory stimulation.

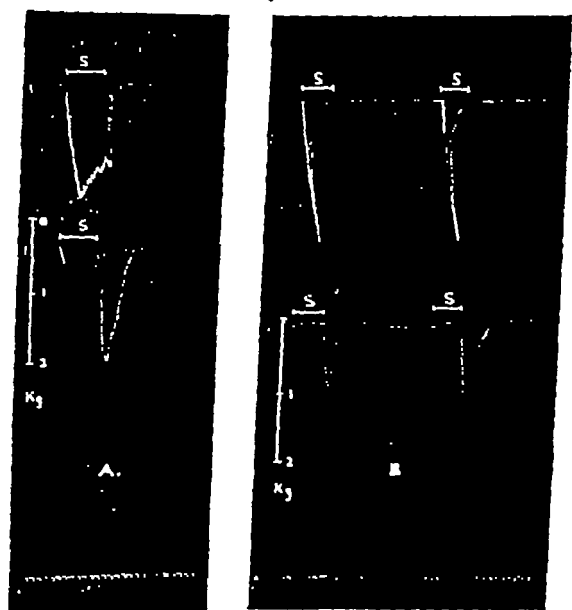


FIG 4—Records as in fig 1 Section A marked rebound contraction after ipsilateral sensory stimulation and reflex quadriceps contraction. Between A and B transection of posterior lumbar roots 5 6 and 8 and all sacral roots. Note persistence of rebound contraction and change in appearance of flexor reflex.

of the fourth and fifth lumbar roots in the production of vasto-crureus rebound. Transection of the sixth posterior lumbar root sufficed either to abolish completely or at least to weaken considerably the three types of rebound contraction which were studied in this muscle. When some measure of rebound persisted after sectioning of the sixth lumbar root, the phenomenon was abolished by additional transection of the seventh root.

It is, therefore, concluded that the discrepancy between Sherrington's and our own observations can be explained by incomplete deafferentation of the vasto-crureus in the older experiments. The integrity of the afferent reflex pathway appears to be indispensable for the occurrence of the rebound phenomenon. Our experiments do not throw any light on the explanations advanced by Graham Brown for his studies of the rebound phenomena in the gastrocnemius muscle.

The conception of the dependence of the rebound phenomena in the quadriceps muscle on the integrity of the proprioceptive reflex arc is further supported by the relationship between the previous degree of muscle stretch and the subsequent rebound contraction. It was shown that increasing the pre-stimulatory stretching load on the muscle increased the extent of the rebound contraction which followed the cessation of afferent stimulation.

The inferences drawn from the present observations are in accord with the known function of the muscle stretch receptors and their primary importance for the initiation of the myotatic reflex, which prevents further impact of the stretching force on the muscle spindles lying in parallel with the contractile elements [Fulton and Pi-Süner, 1928].

If a stimulus inhibitory to a tonic muscle with high activity of the myotatic reflex arc reaches the spinal cord, the myotatic reflex will be suppressed because of the predominance of nociceptive stimulation over pre-existing excitatory states. The suppression of the myotatic reflex, as shown by the frequent relaxation of the muscle, inevitably subjects the muscle-spindles to a higher degree of stretch. If now the inhibitory stimulation be discontinued, the stretch reflex will be operative again and produce a powerful reflex rebound contraction. Sherrington drew attention to the fact, which our observations amply confirm, that the post-inhibitory rebound contraction is always rapid in onset.

Similar considerations would apply to rebound following reflex muscle contraction. When a muscle under tonic innervation is made reflexly to contract, the muscle-spindles are relieved of stretch by the contraction of the muscle-fibres, under conventional recording conditions the potential energy of the myograph would be increased. On the cessation of stimulation the myograph, while returning to its resting level, would subject the muscle-spindles to renewed stretch, thus initiating a powerful post-excitatory rebound contraction.

the sixth and seventh lumbar root (fig 6) This figure also shows that, under these conditions, after-discharge following the cessation of stimulation completely disappeared

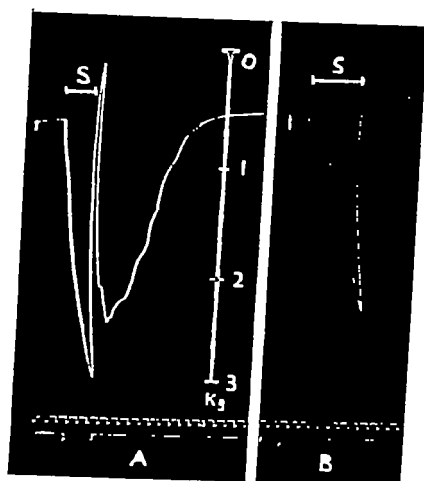


Fig 6—Records from above downwards contraction of left quadriceps muscle, time in sec, signal Horizontal bar marked S indicates central stimulation of right popliteal nerve (70 cyc/sec, 10.0 msec pulse duration, 25 v) Between A and B division of the sixth and seventh lumbar posterior root Note crossed extensor reflex persists, rebound contraction abolished.

### DISCUSSION

These experiments clearly show that rebound contraction in the quadriceps muscle of the decerebrate cat depends on the integrity of the afferent pathway carrying impulses from the muscle to the spinal cord. Rebound contraction can be completely abolished by transection of the appropriate spinal posterior roots.

The older hypotheses propounded to explain the nature of the rebound phenomenon in terms of a central change from an excitatory to an inhibitory state, or in terms of excitatory or inhibitory nerve-fibres, were based on the assumption of a complete independence of the rebound contraction of afferent impulses arising in the muscle. This conception was founded on the observations of Sherrington on the persistence of rebound contraction in the deafferented vasto-crureus muscle. It must be emphasized that Sherrington found rebound contraction in deafferented muscle inconstant in appearance, feeble, and of less duration than in normally innervated muscle.

The difference between Sherrington's procedure and that reported in this paper lies in the method of deafferentation of the vasto crureus. Sherrington sectioned the fourth, fifth and sixth lumbar posterior nerve roots, the seventh root remained intact.

We have not been able to obtain any evidence of the participation

# THE ACTION OF SOME HUMORAL AGENTS ON THE HORSE INTESTINE By FRANK ALEXANDER From the Department of Physiology, Royal (Dick) Veterinary College, Edinburgh

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## INTRODUCTION

ALTHOUGH the alimentary tract of the horse presents many interesting anatomical features, and despite the fact that disturbances of this system account for a large proportion of disorders in the adult animal, few investigations into the mechanics of the intestinal tract have been carried out. Some observations on intestinal movement were made by Colin [1886], who found that waves, described as antiperistaltic, were common in the small intestine of the horse. Unfortunately, his observations were conducted on the intestine of the freshly killed horse without any precautions to prevent drying and cooling. More recently Tanaka and Ohkuho [1940], using the method of Magnus, investigated the action of adrenaline on the ileum of the horse and found that it caused a contraction. Since the observations of Colin and the experiments of Tanaka and Ohkuho gave somewhat unexpected results, it seemed important to extend their studies. An investigation was therefore undertaken into the effects of adrenaline, acetyl choline, histamine and posterior pituitary extract on the activity of the horse's intestine.

## METHODS

Pieces of gut, about 20 cm in length, were taken from a horse immediately after destruction and put in cold Tyrode's solution. Strips of about  $1 \times 10$  cm were cut from the larger pieces and suspended in Tyrode's solution in a bath of 100 c.c. capacity. The strips were taken from the following regions: ileum, caecum, large colon, small colon and rectum. The strips of ileum were cut in a longitudinal direction, great difficulty was experienced in getting responses from strips cut in a circular fashion despite the use of the technique recommended by Sialuhs and Sollmann [1927]. The anatomical arrangement of the muscle-fibres in the large intestine facilitated the separation of the longitudinal and circular fibres, the former being gathered into taeniae [Sisson, 1921]. Strips of taenia and circular fibres were studied separately. The muscle layers of the rectum were easily dissected free from each other, and from mucous membrane, into circular and longitudinal bundles.

## SUMMARY

1 Three types of reflex rebound contractions were studied in the quadriceps muscle of decerebrate cats rebound following reflex inhibition, rebound after reflex contraction caused by ipsilateral afferent stimulation, and rebound after reflex contraction caused by contralateral afferent stimulation

2 The strength of rebound contractions following reflex inhibition and reflex contraction after ipsilateral sensory stimulation increased with an augmentation of the stretching force previously applied to the muscle

3 These types of rebound contractions could not be produced after complete deafferentation of the muscle Transection of the sixth posterior lumbar root usually sufficed to abolish rebound, in those cases where rebound persisted, although decreased in force and inconstant, transection of the seventh posterior lumbar root finally abolished it

4 There was no evidence that the fourth and fifth lumbar posterior root participated in the production of quadriceps rebound

5 Reflex rebound, under the conditions studied, is explained as a phenomenon originating in the proprioceptors of the muscle

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ascending colon, and the aorta rapidly dissected from its attachments to the roof of the abdomen. This allowed the liver, stomach and intestines to be removed from the body by cutting above the œsophageal ligature and below the colic. These organs were placed in a dish of Tyrode's solution while the remaining operations were completed.

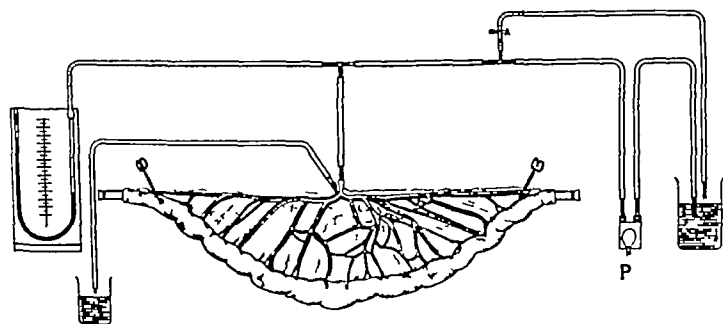


FIG 1—The arrangement for perfusing the isolated horse intestine  
P pump and valves

Cannulæ were introduced into the cranial mesenteric artery and into the common trunk of the portal vein. After placing a ligature round the anastomosis between the duodenal branch of the cœliac and first intestinal arteries, the liver and stomach were removed and the intestines placed in a heated box and perfused in a manner similar to that used for horse intestine.

## RESULTS

### *Intestinal Strips*

The results of the experiments on intestinal strips are summarised in Table I. The most remarkable of these was the marked increase in tone produced by adrenaline on the ileum (fig 2) and circular fibres of the large intestine (fig 3, A). This effect was reversed by ergotoxine (figs 2 and 3, A).

Two cases, in a series of fifty experiments on the horse ileum, occurred in which adrenaline caused inhibition of rhythmic contractions without any increase in tone. Strips of the circular muscle of large and small colon were not so consistent in their response to adrenaline, inhibition of rhythmic contractions occurred in about 25 per cent of preparations from the large colon, and there was no response to adrenaline in about 40 per cent of preparations from the small colon.

Strips cut from the region of the pyloric and ileocæcal sphincters were suspended in the bath and their response to drugs tested. In the few instances in which a response was obtained, the effect of adrenaline was to increase the tone.

Solutions of the drugs were prepared by diluting a 1/1000 solution of adrenaline in Tyrode's solution and by making fresh solutions of acetyl choline and histamine acid phosphate. A preparation of posterior pituitary (Burroughs Wellcome) containing 10 units per c c was used.

Before any tests were made each strip was suspended in the bath for at least 30 minutes. The drug under investigation was added to the bath in increasing concentration until a measurable response was obtained. The dose required to produce this effect is shown in the appropriate figure. When an effective concentration had been reached, a dose ten times as big was added to make sure that the response first obtained was not a specific response due to the presence of the drug in high dilution. Gruber [1922] found that adrenaline in high dilution caused contraction of the frog's intestine whereas stronger solutions gave the opposite effect.

### *Perfusion Technique*

In order to study the effect of these drugs on intestinal propulsion, other experiments were devised in which the gut was maintained in a viable state by the perfusion of its blood-vessels with physiological saline solutions or blood.

A length (about 1 m) of ileum or small colon, supplied by one intestinal artery, was removed from a freshly killed horse. The artery and vein were cannulated and the open ends of the intestine tied over glass tubes of suitable diameter. The loop of bowel was then laid on a sheet of perspex and placed in a heated box the temperature of which was thermostatically controlled at 36-38° C. All the blood was washed out of the organ by perfusing with Tyrode's solution at 37° C. In the early experiments the perfusion pressure was controlled by raising or lowering a bottle fitted with a Mariotte tube, later this was replaced by a pump. The perfusion apparatus is illustrated by fig 1.

Tyrode's solution, blood, washed red cells and plasma were used as perfusion fluids. When defibrinated blood was employed, some difficulty was experienced from the presence of vasotonins and, while it was possible to remove them by first passing the blood through a separate intestinal loop, it was preferable to overcome this obstacle by using chlorazol fast pink as anti-coagulant.

Experiments were conducted in which the effect of different perfusion fluids on the propulsion of boluses, seeds and fluids were observed. The action of drugs on intestinal movements was measured by recording the response on a smoked drum using a volume recorder connected to the glass tube in the end of the segment.

To perfuse the cat's bowel, a cat was etherised and bled by severing the carotid arteries, ligatures were placed round the oesophagus and

from the large colon or cæcum Further, the dose of adrenaline required to inhibit rhythmic contractions of cæcal and large colon

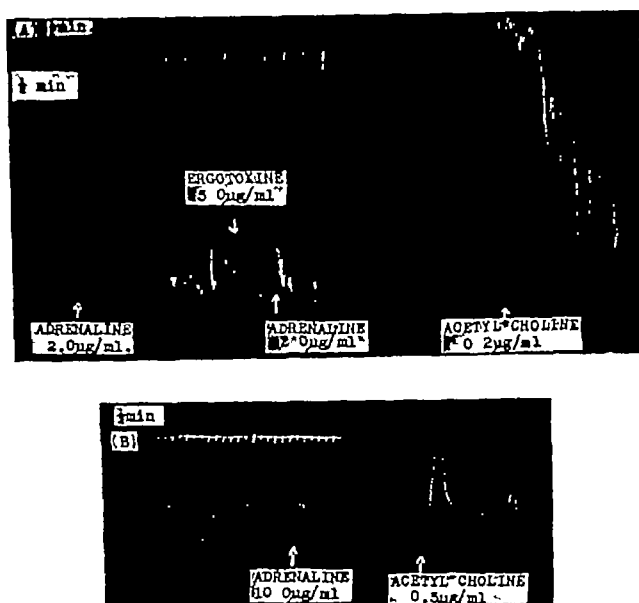


FIG 3—A. The action of adrenaline and acetyl choline on a circular strip of large colon.

B The action of adrenaline and acetyl choline on a strip of large colon tæniæ

tæniæ was greater than needed to inhibit those of the small colon tæniæ (fig 3, B)

The responses to acetyl choline (fig 3) and histamine (fig 4) were consistent, and easily elicited from all preparations

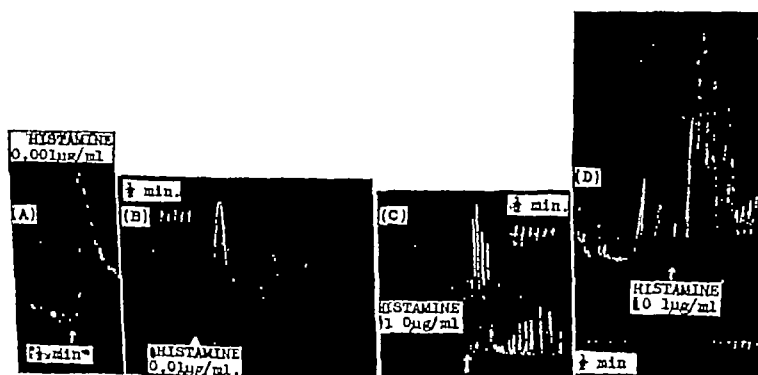


FIG 4—The action of histamine on (A) ileum, (B) large colon tæniæ, (C) small colon tæniæ, (D) rectum

TABLE I—THE EFFECT OF ADRENALINE, ACETYL CHOLINE, HISTAMINE AND POSTERIOR PITUITARY EXTRACT ON INTESTINAL STRIPS FROM THE HORSE.

Drug	Tissue	Effect	
		Tone	Rhythm
Adrenaline	Ileum	Increased	Unchanged
	Caecum	Increased	Inhibited
	Circular strips	Unchanged	Inhibited
	Tænia	Increased	Increased
	Large colon	Unchanged	Inhibited
	Circular strips	Increased	Increased
	Tænia	Unchanged	Inhibited
	Small colon	Increased	Increased
	Circular strips	Unchanged	Inhibited
	Tænia	Increased	Increased
Acetylcholine	Rectum	Increased	Increased
	Circular strips	Unchanged	Inhibited
Histamine	Longitudinal strips	Increased	Increased
	All	Increased	Increased
Posterior pituitary extract	All	Increased	Increased
	All	Unchanged	Unchanged

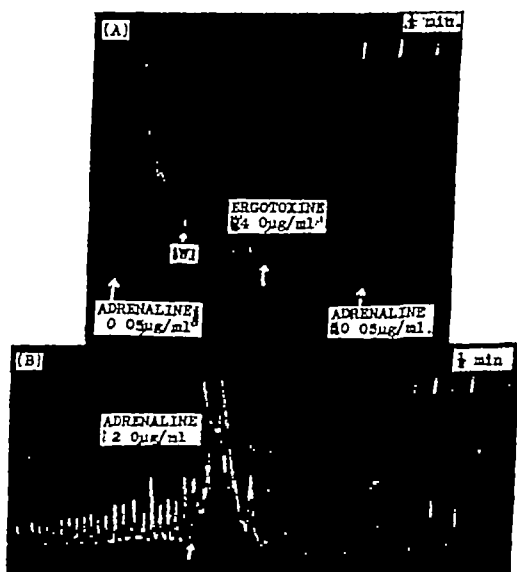


FIG 2—A Usual effect of adrenaline on a strip of ileum  
 B Diphasic action of adrenaline on the ileum seen in a few cases

It was noted that the rate of rhythmic contraction in strips of tænia from the small colon was about three times as fast as in strips of tænia

	Oral outflow	Aboral outflow
16 35	Perfusion begun	
16 40	180 cc	30 cc
16 45	10 cc	15 cc
16 50	10 cc	55 cc
16 55	0 cc	55 cc
17 00	5 cc	10 cc

### *Investigation of the Perfusion Fluid*

To determine the fraction of blood responsible for these propulsive movements, red cells were separated by centrifugation, washed in Tyrode's solution and restored to their original volume with Tyrode's solution. When this solution was used for the perfusion of the ileum, the same movements were observed as with defibrinated blood as the perfusing fluid, similar propulsion took place. Perfusion with plasma produced an increase in movements which rarely persisted for more than 30 minutes, and was much weaker than that produced by perfusion with washed red cells. The movements produced by perfusion with blood or washed red cells were maintained for at least 3 hours.

Graphic records of the variations in intraluminal pressure produced by perfusion with Tyrode's solution, plasma and washed red cells are shown in fig. 6

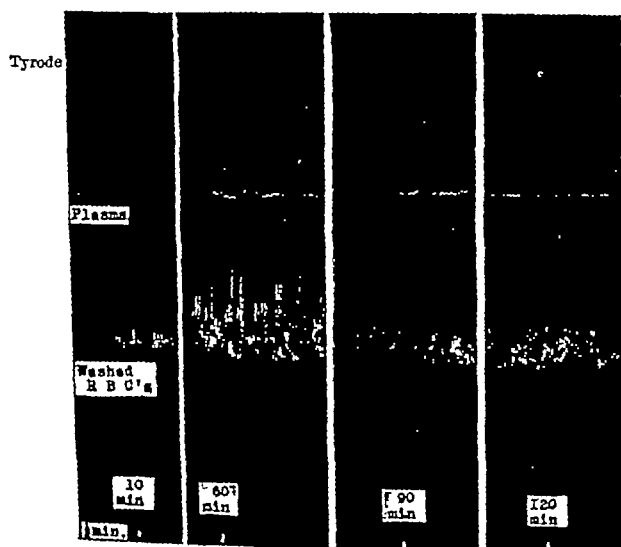


FIG. 6 — The effect of different perfusion fluids on the movements of the perfused ileum. The time from the start of perfusion is shown in minutes

### Perfusion Experiments

When loops of horse ileum were perfused with oxygenated Tyrode's solution, slow, inco-ordinated, pendular movements and feeble waves of contraction, running orally and aborally, were observed. These movements did not cause any progression of objects placed in the lumen of the bowel.

Perfusion of the bowel with defibrinated blood induced strong movements of the following types: peristalsis, antiperistalsis, pendular movements, and a spiral twisting of the gut on its mesenteric border. The predominant type of movement was a progressive segmentation, no peristaltic rushes were seen. These movements produced an interesting variety of effects on different objects placed in the lumen. Boluses of cotton-wool and soft paraffin, about 3 cm. in diameter, were moved to and fro, the overall progression was towards the oral end of the segment. The following experiment illustrates this phenomenon —

#### Expt 38

Perfusion fluid—defibrinated blood

Perfusion pressure—70 mm Hg

14 15 Bolus placed in the middle of the loop of bowel

14 30 Bolus had progressed 10 cm. towards oral end

14 45 Bolus had progressed 15 cm. towards oral end

Seeds and inert particles were propelled both orally and aborally, but more particles were propelled towards the aboral end of the bowel than the reverse. This is shown graphically in fig. 5.

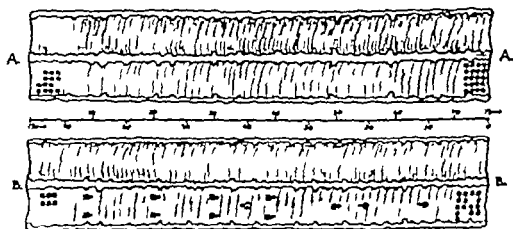


FIG. 5—A shows the seeds placed at each end of a loop of ileum. B-B shows their position 2 hours later, the intestine having been perfused with defibrinated blood for this period. The oral end of the loop is on the left.

In other experiments a glass tube, closed by rubber tubing and a spring clip, was introduced through the bung (fig. 1) at each end of the segment. The lumen of the ileum was filled with normal saline solution. When the two ends were simultaneously unclipped and the outflow measured, it was found that saline was expelled at each end of the segment. The following experiment illustrates this finding —

	Oral outflow	Aboral outflow
16 35	Perfusion begun	
16 40	180 cc	30 cc
16 45	10 cc	15 cc
16 50	10 cc	55 cc
16 55	0 cc	55 cc
17 00	5 cc	10 cc

### *Investigation of the Perfusion Fluid*

To determine the fraction of blood responsible for these propulsive movements, red cells were separated by centrifugation, washed in Tyrode's solution and restored to their original volume with Tyrode's solution. When this solution was used for the perfusion of the ileum, the same movements were observed as with defibrinated blood as the perfusing fluid, similar propulsion took place. Perfusion with plasma produced an increase in movements which rarely persisted for more than 30 minutes, and was much weaker than that produced by perfusion with washed red cells. The movements produced by perfusion with blood or washed red cells were maintained for at least 3 hours.

Graphic records of the variations in intraluminal pressure produced by perfusion with Tyrode's solution, plasma and washed red cells are shown in fig. 6.

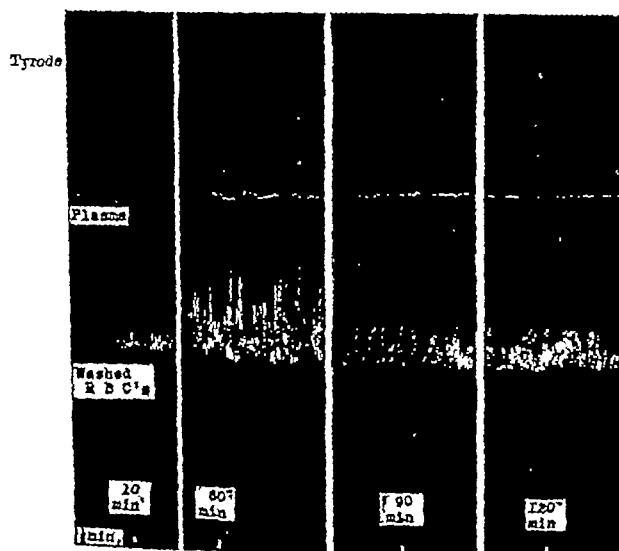


FIG. 6.—The effect of different perfusion fluids on the movements of the perfused ileum. The time from the start of perfusion is shown in minutes.

### *Perfusion of the Ileum of the Cat*

In this species propulsion occurred with Tyrode's solution as the perfusing fluid. Propulsion was always in an aboral direction. The following experiment was typical —

#### *Ileum of Cat Perfused with Tyrode's Solution through the Cranial Mesenteric Artery*

11 45	Pea introduced into duodenum
12 05	Pea had progressed 5 cm aborally
12 10	" " " 9 cm "
12 25	" " " 20 cm "
12 40	" " " 20 cm "

### *Perfusion of the Small Colon*

When this organ was perfused with oxygenated Tyrode's solution weak movements were observed. The predominant type was a rippling movement of the haustra, which seemed to have the effect of kneading the contents. Slow peristaltic and antiperistaltic waves and an occasional contraction of the *tænæ* were seen.

Perfusion with blood or a suspension of washed red cells produced the same types of movement but increased in strength. The increase in strength was not so great as in the ileum. These movements did not cause any progression of the normal contents of the small colon or of artificial boluses introduced into the lumen.

### *The Effect of Drugs on the Perfused Bowel*

The various drugs were injected into the arterial cannula of the perfused bowel. The total volume of the perfusing fluid was about one litre. The results of these experiments are given in Table II. The

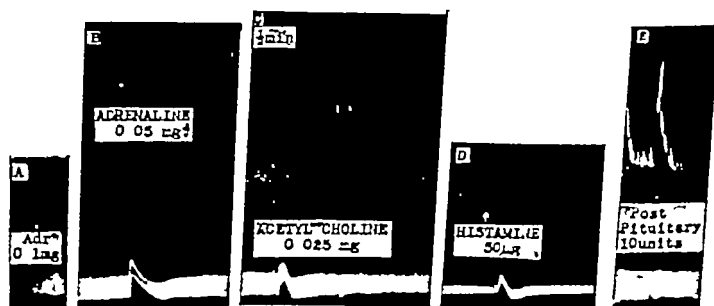


FIG 7 — (A) the effect of adrenaline on the Tyrode perfused ileum (B), (C) (D), (E), the effect of various drugs on the blood perfused ileum



effects of drugs on the perfused ileum are shown in fig 7, and on the perfused small colon in fig 8

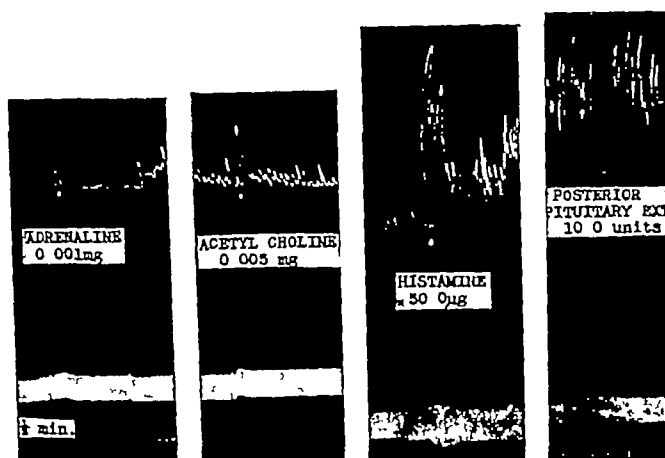


FIG 8 —The effect of various drugs on the blood perfused small colon.

TABLE II —THE EFFECT OF DRUGS ON THE PERFUSED BOWEL OF THE HORSE

Drug	Organ	Perfusion fluid	Effect	
			Tone	Rhythm
Adrenaline	Ileum	Tyrod's Blood	Increased	Unchanged
	Small colon	Blood	Decreased	Inhibited
Acetyl choline	Ileum	Blood	Increased	Inhibited
	Small colon	Blood	Increased	Increased
Histamine	Ileum	Blood	Increased	Increased
	Small colon	Blood	Increased	Increased
Posterior pituitary extract	Ileum	Blood	Increased	Increased
	Small colon	Blood	Increased	Unchanged
	Small colon	Blood	Decreased	Inhibited

## DISCUSSION

### *Action of Drugs on Intestinal Strips*

The experiments described have confirmed the findings of Tanaka and Ohkuho [1940], and, further, have shown that the excitor effect of

adrenaline on the horse's intestine was not confined to the ileum. This effect of adrenaline was demonstrated on circular strips of cæcum, colon and rectum. It was independent of the tone of the tissue or the concentration of adrenaline. It was important to show the effect independent of these conditions, since Brown and McSwiney [1927] found the action of adrenaline on strips of muscle from the stomach of rabbits varied with their tone. Similarly, Gruber [1922] found dilute solutions of adrenaline caused contraction of the isolated frog intestine whereas concentrated solutions produced inhibition.

It was possible, by adding ergotoxine to the bath, to change the excitor effect of adrenaline on these tissues of the horse into one of inhibition. Though adrenaline has a diphasic effect on the activity of several organs, it has not so far been shown to affect the intestine in this way. A satisfactory explanation of the diphasic action is that first proposed by Dale [1906], who suggested that these viscera may have both excitor and inhibitor adrenergic fibres in their nerve-supply. Acetylcholine produced characteristic effects on the intestinal strips. Histamine, however, had the interesting property of inducing strong rhythmic contractions, an effect which was particularly marked on strips of tænia. Posterior pituitary extract showed no activity when tested by this method. Since this substance finds some employment clinically for the stimulation of intestinal movement, it is important to test its action on the intestine by other methods.

### *Perfusion Experiments*

The main purpose of the intestinal movements is to mix the ingesta with the intestinal secretions and, having exposed the mixture to the mucosa, to propel the residue aborally. To study these movements satisfactorily it is necessary to devise a preparation in which a length of intestine can be observed as a whole. With this in view, the method of perfusing segments of bowel was developed, and it was hoped this preparation would facilitate the investigation of the factors influencing propulsion.

The perfusion experiments on the ileum showed that unless red blood cells were present in the perfusing fluid propulsion did not occur, and that adequate oxygenation was an important factor in the co-ordination of the bowel movements. The possibility of this feature being peculiar to the horse was at once considered, and similar experiments were performed on the ileum of the cat. These experiments showed that perfusion with oxygenated Tyrode's solution enabled the ileum of the cat to propel objects placed in the lumen.

It is clear from these experiments that differences exist between the factors controlling intestinal propulsion in the different species. This

finding is supported by the work of van Liere and his co-workers [1943], who found that anoxia decreased intestinal propulsion in mice but had no effect in dogs. Similarly, Carnot and Glenard [1912] and Glenard [1913] observed peristaltic movements in the Locke perfused intestine of the dog and studied the progress of objects through the lumen.

It seemed possible, from these findings, that the dietetic habits of the species might determine the degree to which anoxia interfered with intestinal propulsion, herbivores being more sensitive to this condition than carnivores. Moreover, Alvarez [1937] found that anoxæmia completely disrupted the peristaltic rush in rabbits. This finding, however, was not in agreement with the results obtained by von Oettingen, Sollmann and Ishikawa [1928]. These latter authors found that long segments of rabbit ileum, suspended in oxygenated Locke solution, propelled various liquids along the lumen by means of peristaltic waves. They stated, however, that an intraluminal pressure of 15 mm. water was necessary to elicit propulsion, hence the ensuing peristalsis might well have been due to the simple distension of the gut, as in Trendelenburg's preparation. It would have been interesting if von Oettingen and his colleagues had investigated the effect of increasing the intraluminal pressure to similar levels at the caudal end of the segment.

### *Analysis of Movements of Perfused Ileum*

The movements of the perfused ileum were difficult to analyse. The predominant type was a progressive segmentation which frequently merged into a slow wave of peristalsis. These movements were shown to propel the intestinal contents both orally and aborally, a somewhat unusual observation which is in agreement, however, with the early work of Colin [1886], who, observing the bowel movements in recently killed horses, noted the occurrence of rings of constriction, running both orally and aborally, which pushed the contents of the small intestine from duodenum to ileum and ileum to duodenum.

Since the experiments on the ileum of the cat, performed with an identical perfusion technique, did not show any antiperistaltic propulsion, it seemed unlikely that the antiperistalsis recorded on the ileum of the horse was an artifact. A possible function of this movement might be the more complete mixing of the intestinal contents, and it was noted that mixtures of gum acacia and carbon were soon evenly spread through the perfused loop. Similarly, London [1911] found that no matter the size of meal consumed, it was evenly distributed over the ileum so that all parts shared in the work of absorption. In the experiments on the perfused ileum of the horse, the trend of propulsion was in an anal direction except when large boluses were present. In this case the

purpose of the antiperistalsis might have been to reduce the smaller state of division, since normally the ileum never contains particles

### *Movements of the Small Colon*

The movements of the perfused small colon were confined, exclusively, to the haustra. A rolling movement of the haustra was the most common, although both peristaltic and antiperistaltic movements were observed. Contraction of the antimesenteric tænia was occasionally observed.

In view of the results obtained on the ileum it was surprising that perfusion with neither Tyrode's solution nor blood produced any noticeable degree of propulsion in the small colon, no matter whether the contents or artificial boluses were in the lumen. The work of Lill and Magnus [1905], however, indicated that the colon of the rabbit possessed an intrinsic propelling mechanism. They found that movements of the distal colon of the rabbit, suspended in physiological solution, pushed faecal pellets from the cranial to the caudal end. In the distal colon and small colon are analogous structures, it seemed that the failure of the isolated small colon of the horse to propel its contents might be due to (a) separation from the central nervous system, (b) disruption of continuity of the alimentary tract, (c) injury to sensitive structures caused by the experimental manipulations. These points require further elucidation. It may be noted that Alving [1939] made the interesting suggestion that the functions of the stomach and colon are controlled by the central nervous system to a much greater degree than is the small intestine.

### *Effect of Drugs on the Perfused Bowel*

The experiments on the effect of drugs on the perfused intestine showed similar results to those observed on the Magnus preparation, with the exception of adrenaline. This substance caused contraction of the isolated strip and Tyrode's perfused ileum, whereas it completely inhibited the contractions of the blood-perfused ileum. These results were somewhat difficult to reconcile, a possible explanation might be that, as the contractions of the blood-perfused ileum were propulsive in nature, they were, to some extent, co-ordinated by nervous mechanisms. The effect of adrenaline in stopping them might have been due to its action on such a co-ordinating mechanism. In the case of the isolated strip and Tyrode's perfused ileum the action might have been solely on the muscle, due to the co-ordinating mechanism being in abeyance as a result of anoxia.

Of the substances studied, the only one which increased the activity

of isolated strips, blood-perfused ileum and blood-perfused small colon was histamine. The results of the experiments with posterior pituitary extract offered no support for the use of this drug to stimulate intestinal movements. Indeed, its use seemed to be contra-indicated.

The results of the experiments on the isolated tænia, in which tæniæ from the small colon showed a faster rate of contraction than those from the large colon and cæcum, do not accord with Alvarez' [1939] gradient theory of intestinal activity. A possible explanation of this, however, may lie in the histology of the tænia. Cæcal tæniæ and tæniæ of the large colon consist mainly of fibrous tissue with little smooth muscle, whereas tæniæ from the small colon are composed almost entirely of smooth muscle [Sisson, 1921]. Nevertheless, considering the activity shown by strips of tæniæ, it seems unlikely that their sole function is to support the circular fibres as suggested by Auston [1937].

#### SUMMARY

The actions of adrenaline, acetyl choline, histamine and posterior pituitary extract on the intestine of the horse have been investigated. The Magnus preparation and a perfusion technique were used. The perfusion method is described in detail.

Propulsive movements of the horse ileum were only produced when the perfusion fluid contained red blood cells. The cat ileum performed propelling movements when perfused with Tyrode's solution.

In the horse ileum both oral and aboral propulsion occurred, aboral propulsion predominating. The small colon did not propel its contents even when perfused with blood.

Excepting adrenaline, the various humoral agents gave similar effects on the Magnus preparation and on the perfused bowel. Adrenaline produced a rise in tone with little change in the rhythmic contractions in isolated strips of ileum, Tyrode-perfused ileum and isolated strips of the circular muscle of the large intestine, whereas it inhibited the movements of the blood-perfused ileum and small colon. Acetyl choline and histamine caused a rise in tone and usually increased the rate of rhythmic contractions. Posterior pituitary extract produced little effect on the various preparations of intestine.

#### ACKNOWLEDGMENTS

I wish to thank Professor J. H. Gaddum, F.R.S., for his advice and encouragement, Mr. D. M. Douglas for criticising the manuscript, Professor W. M. Mitchell for laboratory accommodation, and the Agricultural Research Council for a grant to carry out this work.

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THE ACTION OF CARBON DIOXIDE ON ISOLATED PERFUSED DOG LUNGS By HELEN N DUKE From the Department of Physiology, University of Edinburgh

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BINET and BOURLIERE [1941] found that inhalation of carbon dioxide produced an increase of pulmonary arterial pressure in isolated perfused dog lungs, and an increase of lung blood-volume if the concentration of  $\text{CO}_2$  was less than 50 per cent. Previous investigators [Fuhner and Starling, 1913] had attributed the rise of pulmonary arterial pressure produced by  $\text{CO}_2$  in dog heart-lung preparations to cardiac failure. Carbon dioxide has also been found to have a vasoconstrictor action in isolated perfused cat lungs [Lohr, 1924] and in the whole anaesthetized cat [Von Euler and Liljestrand, 1946], but other investigators using the cat heart-lung preparation [Drinker, Churchill and Ferry, 1926] or direct microscopy in anaesthetized cats [Wearn, Ernestine, Bromer, Barr, German and Zschiesche, 1934] concluded that  $\text{CO}_2$  had no action on the lung blood-vessels.

Some experiments performed in this laboratory [Daly, Duke, Fegler and Roughton, 1945] suggested that the effects of  $\text{CO}_2$  on dog lungs might be somewhat different from those observed by other workers, and it was decided that further investigation was required.

#### METHODS

The experiments were performed on isolated perfused lungs of dogs using a technique similar to that described by Berry and Daly [1931]. Dogs (8-20 kg body-weight) were killed by bleeding from the femoral artery which was exposed under local (Novocaine) anaesthesia, and the pulmonary artery, left auricle and trachea were immediately cannulated. Four animals were anaesthetized with chloralose (0.1 g/kg) before bleeding. The blood was collected and clotting prevented by the addition of heparin (BDH) or Liquemin (Roche) 1000 IU/100 cc. The lungs were perfused with the animal's own blood at constant volume inflow through the pulmonary artery by means of a Dale-Schuster pump. The venous return of the lungs was drained from the left auricle cannula into a reservoir which fed the pump. In the majority of experiments the blood inflow was adjusted to give an initial pulmonary arterial

pressure of 30 cm blood, but in others lower pressures were used (down to 7.5 cm)

Of the four animals under chloralose anaesthesia two were kept alive by artificial respiration during the preparations for perfusion. The left auricle was cannulated, a ligature was placed round the pulmonary artery and the animal was bled from the carotid artery directly into the venous reservoir. While positive pressure ventilation was still maintained, a cannula was inserted into the pulmonary artery and perfusion was begun. Perfusion was continued while the lungs were removed from the body and transferred to the negative pressure chamber. This method ensured that the circulation through the lungs was interrupted for only 2 to 5 minutes (instead of 30 minutes or longer).

The pulmonary arterial pressure and the volume of blood in the venous reservoir were recorded in all experiments with the type of apparatus recommended by Daly [1938]. Changes in pressure indicated parallel changes in the resistance of the pulmonary blood-vessels, while changes in the venous reservoir volume indicated inverse changes in the lung blood-volume [Daly, 1928].

The lungs were ventilated either by positive pressure using a Starling "Ideal" pump, or by means of extrapulmonary negative pressure variations (ca -2 to -12 cm H<sub>2</sub>O, 12-18 min).

*Changes in the Ventilating Gas Mixture*—The gas mixtures used were all from commercial cylinders, containing either 5 or 10 per cent CO<sub>2</sub> in air (the error found on analysis was within 1 vol per cent). When positive pressure ventilation was used, a Douglas bag containing the appropriate gas mixture was attached to the input of the Starling "Ideal" pump. Control gas analyses showed that there was no detectable leakage of CO<sub>2</sub> from the bag over the experimental period for which it was required. When negative pressure ventilation was used, the carcass of the dog with the lungs *in situ* was enclosed in a sealed chamber (of approximately 70 litres capacity) and the trachea was connected to a T-piece containing Siebe-Gorman inspiratory and expiratory valves, the inlet and outlet of which were led through the chamber wall. Arrangements were made for temporarily connecting the tube from the expiratory valve to a small recording spirometer so that the volume of the expired gas could be recorded for 2 to 3 respirations at frequent intervals. This measured the tidal air, and it was of the order of 150-250 cc at the beginning of the experiment. A Douglas bag containing a test gas mixture could be connected to the inspiratory side when desired.

This method of administering gas mixtures had two advantages: the dead space of the apparatus was minimized (to about 100 cc), and it was possible to maintain a constant concentration of CO<sub>2</sub> in the ventilating gas mixture. For this latter purpose a closed system had not been found to be practicable.



The observations were continued for 4 to 6 hours of perfusion, depending on the condition of the preparation. No observations were made in lungs after the onset of oedema, which might occur after longer periods of perfusion.

Blood samples were taken from the pulmonary arterial tubing into glass tonometers, which had been filled with mercury. The blood oxygen and carbon dioxide content was estimated by the method of Peters and Van Slyke [1932]. The analyses were made 2 to 6 hours after the samples had been taken, during which time the tonometers were stored in a refrigerator.

### RESULTS

*Responses to an Increased Concentration of CO<sub>2</sub> in the Ventilating Gas Mixture*—A change in the ventilating gas mixture from air to air containing 5 per cent CO<sub>2</sub> (42 tests in 24 experiments) was followed, after a latent period of 10 to 30 seconds, by a decrease in lung blood-volume. The loss of blood from the lungs was at first rapid, and then gradually reached an asymptote after a period which varied from 16 to 46 minutes, although the administration of 5 per cent CO<sub>2</sub> was usually continued for a longer period. The total diminution of lung blood-volume was of the order of 20 to 30 c.c. (see Table I), but some preparations were less sensitive (see experiments 2 and 6, Table I). Fig. 1 shows the effect of 5 per cent CO<sub>2</sub> on the pulmonary arterial pressure.

TABLE I—EFFECTS ON THE PULMONARY BLOOD VESSELS OF CHANGING THE VENTILATING GAS MIXTURES FROM AIR TO AIR CONTAINING 5 PER CENT CO<sub>2</sub>

No of Expt	Weight of dog kg	Time from start of perfusion to change	Duration of administration of 5 per cent CO <sub>2</sub>	L B V diminution, c.c.	Maximum P.A.p. change cm blood
*2	17	1 hr 12 min	50 min	16	+2.5
*3	12	2 hr 54 min	1 hr 13 min	7.5	+3.0
6	12.8	3 hr 38 min	44 min	31	+2.3
*13	10.3	1 hr 45 min	1 hr 3 min	13	+1.0
14	8	3 hr 25 min	42 min	32	+1.0
15	12.8	55 min	36 min	21	None
16	13.8	1 hr 2 min	48 min	27	-1.5
17	13.0	57 min	42 min	24	-1.5
18	9.0	53 min	41 min	18.5	-0.5
		57 min	29 min	23	None
		58 min	21 min	19	+0.75
20	21.0	2 hr 18 min	21 min	9	+0.5
		3 hr 45 min	21 min	9	+0.5

\* Positive pressure ventilation

and lung blood-volume in four experiments, and fig 2 is a tracing obtained in one of the experiments shown in fig 1 Similar results

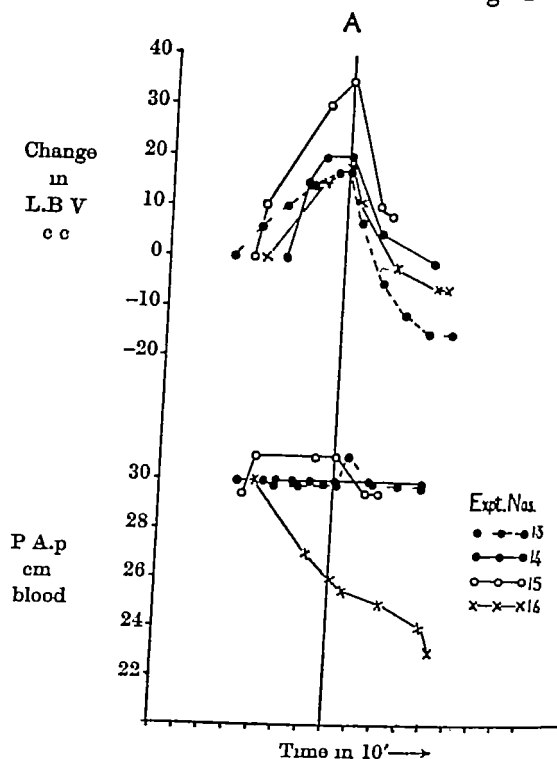


Fig 1—The effect of changing the ventilating gas mixture from air to air containing 5 per cent  $\text{CO}_2$ . The initial L.B.V. level is arbitrarily taken as zero. The vertical line (A) marks the time of the change of gas mixture in each experiment. Positive pressure ventilation was used in experiment No. 13. In Nos. 14, 15 and 16 the lungs were ventilated by negative pressure. L.B.V. = lung blood volume. P.A.p. = pulmonary arterial pressure.

TABLE II—EFFECTS ON THE PULMONARY BLOOD-VESSELS OF CHANGING THE VENTILATING GAS MIXTURE FROM AIR CONTAINING 5 PER CENT  $\text{CO}_2$  TO AIR CONTAINING 10 PER CENT  $\text{CO}_2$ .

No of Expt	Weight of dog, kg	Time from start of perfusion to change	Duration of administration of 10 per cent $\text{CO}_2$	L.B.V. diminution, c c	Maximum P.A.p. change cm blood
*13	10.3	4 hr 7 min	18	19	+0.5
15	12.8	1 hr 50 min	31	65	+2.25
16	13.8	1 hr 39 min	23	30	-1.5
17	13.0	1 hr 34 min	14	27.5	+0.5
18	9.0	1 hr 26 min	25	15	None

\* Positive pressure ventilation

were obtained when the control respiratory mixture was oxygen and the test mixture 5 per cent  $\text{CO}_2$  in  $\text{O}_2$ . In 5 experiments the gas mixture was changed from air containing 5 per cent  $\text{CO}_2$  to air containing 10 per cent  $\text{CO}_2$ , and a further diminution of lung blood-volume which varied from 15 to 65 c c then occurred (see Table II and fig 3). These values do not represent the maximum change of lung blood-volume which could have been produced by 10 per cent  $\text{CO}_2$ , since, in every test, ventilation with 10 per cent  $\text{CO}_2$  was stopped while the lung blood-volume was still falling. With pure  $\text{CO}_2$ , which was administered for only a short period (1-3 minutes) in 3 experiments, the lung blood-volume diminished more rapidly than with lower concentrations, but the full effect of pure  $\text{CO}_2$  was again not measured, because the period of exposure was purposely restricted.

These results were independent of the use of chloralose anaesthesia in the preparation, or of the use of positive or negative pressure ventilation.

In only 2 experiments out of 26 was  $\text{CO}_2$  without effect on the lung blood-volume. On one of those occasions there had been an accidental obstruction to the venous outflow for the first hour of perfusion and the lungs were coming oedematous. On the other occasion the extra-pulmonary negative pressure had been purposely increased much beyond that normally used, and the lungs were rapidly taking up blood.

When the lungs were ventilated by negative pressure, alterations in the  $\text{CO}_2$  concentration of the ventilating gas mixtures did not produce changes in the tidal air, and control tests made with Douglas bags filled with air instead of a test gas mixture gave negative results (see fig 4). Thus mechanical sources of error due to changes in bronchial calibre and alterations of intra-alveolar pressure were excluded.

Changes in pulmonary arterial pressure were not so consistently observed as were changes in lung blood-volume. A slight initial increase of pulmonary arterial pressure, which was seldom maintained during the whole period of administration of the gas mixture containing excess  $\text{CO}_2$ , was observed in approximately 50 per cent of the tests. Fig 4 shows an increase of pulmonary arterial pressure following ventilation of the lungs with 5 per cent  $\text{CO}_2$  in air (from air). In another experiment the pulmonary arterial pressure subsequently fell to below its original level 10 minutes after the change in gas mixture without any effect on the rate of diminution of lung blood-volume. An increase of P A p in response to 5 per cent  $\text{CO}_2$  was seen in all the experiments in which positive pressure ventilation was used, and in these the tidal air was approximately 20-30 c c/kg body-weight. Of 16 tests made in 9 consecutive experiments in which negative pressure ventilation was used, 5 pressor responses were obtained. The tidal air was greater than 13 c c/kg body-weight in each of these, but was less than 13 c c/kg body-weight in 9 out of the other 11 tests. Applying the  $\chi^2$

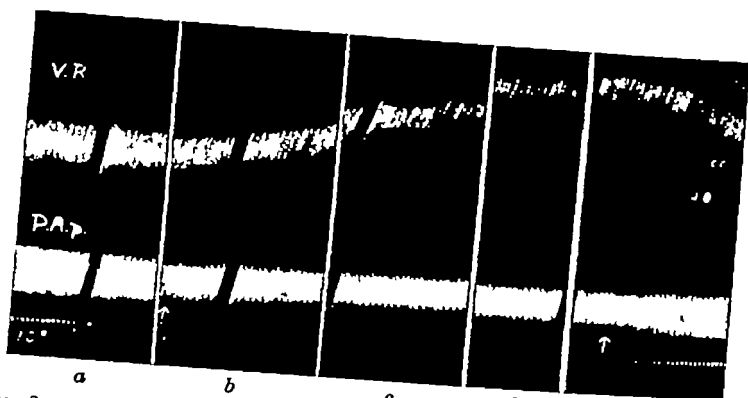


Fig 2—Expt 14. Dog, 80 kg Chloralose (0.1 g/kg) given before bleeding  
 Negative pressure ventilation. Perfusion began 11 45 a.m.  
 a 12 35 p.m. Ventilation with air  
 b 12 37 p.m. Arrow and signal mark time of change to 5 per cent  $\text{CO}_2$  in air  
 The increased volume of blood in the V.R. shows a decrease of L B V  
 c 12 47 p.m. Change to air at signal and arrow  
 d 1 08 p.m. No change in P.A.p., which remained at 30cm blood  
 e 1 16 p.m. V.R. = venous reservoir

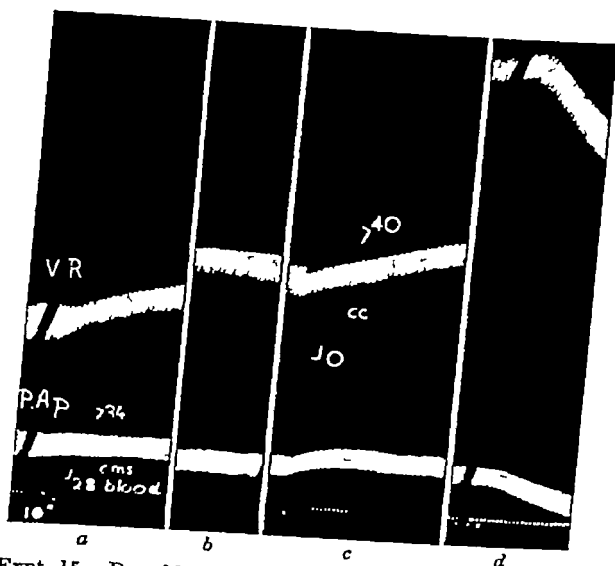


Fig 3—Expt 15 Dog, 12.9 kg Negative pressure ventilation. Perfusion began at 11 30 a.m.  
 a 12 32 p.m. Change from air to air containing 5 per cent  $\text{CO}_2$  (at signal) Tidal air 252 c.c.  
 b 12 56 p.m. Tidal air 215 c.c.  
 c. 1 24 p.m. Change from air containing 5 per cent.  $\text{CO}_2$  to air containing 10 per cent  $\text{CO}_2$  (at signal) Tidal air 200 c.c.  
 d 1 55 p.m. Change from air containing 10 per cent  $\text{CO}_2$  to air (at signal) Tidal air 200 c.c.

test [Bradford Hill, 1945],  $\chi^2 = 6.32$  and  $p$  is less than 0.01. Thus the presence or absence of a pulmonary pressor response may be related to the volume of the tidal air. The concentration of  $\text{CO}_2$  inhaled also determined to some extent whether or not a pressor response was

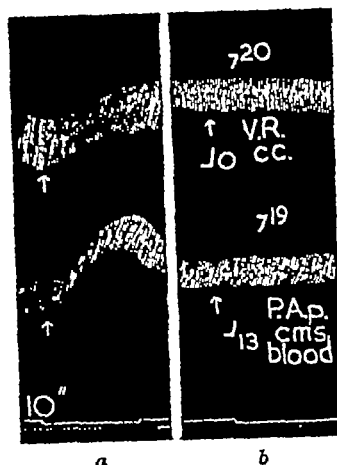


FIG 4.—Expt. 24 Dog, 16.3 kg Chloralose 0.1 g/kg given before bleeding. Circulation through lungs stopped for 2 minutes between death of animal and perfusion. Perfusion started at 12.38 p.m.

a 3.19 p.m. Change from air to air containing 5 per cent  $\text{CO}_2$ . Tidal air 450 c.c.

b 4.17 p.m. Control test with Douglas bag of air. Negative pressure 0 to -16 cm  $\text{H}_2\text{O}$  at a 0 to -10 cm  $\text{H}_2\text{O}$  at b

observed. Thus in fig. 3 no increase of pulmonary arterial pressure followed the administration of 5 per cent  $\text{CO}_2$ , but increasing the concentration of  $\text{CO}_2$  to 10 per cent produced a rise of pulmonary arterial pressure.

These observations suggest that the increase in pulmonary arterial pressure which was sometimes observed was related to the rate of increase of the alveolar concentration of  $\text{CO}_2$ . This was also suggested by Hebb and Nimmo-Smith [1948] as a reason for the differences between their results on dog and *Macacus rhesus* lungs.

The methods used in this series of experiments produced very large changes in blood  $\text{CO}_2$  content. After the lungs had been ventilated with air for about one hour, the concentration of  $\text{CO}_2$  in the pulmonary arterial blood was sometimes as low as 3.0 vol. per cent, while 40 to 50 minutes after changing to 5 per cent  $\text{CO}_2$  in air it was of the order of 30 vol. per cent, and ventilation with 10 per cent  $\text{CO}_2$  for 15 to 30 minutes increased it still further to at least 50 vol. per cent. The oxygen content of the arterial blood remained nearly constant in each experiment (with variations of less than 1 vol. per cent), and varied between 16 and 20 vol. per cent in different experiments. It was not

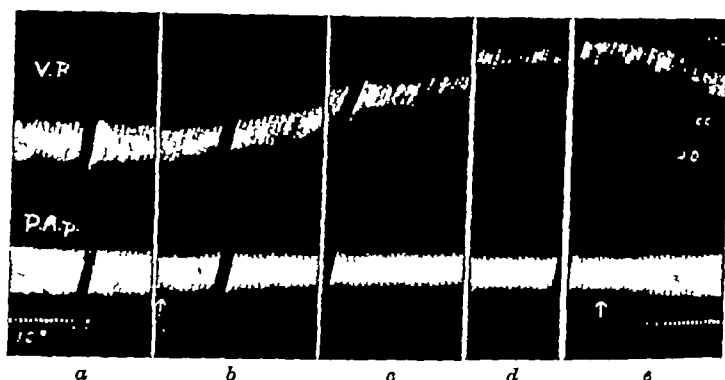


FIG 2—Expt 14 Dog, 80 kg Chloralose (0.1 g/kg) given before bleeding  
Negative pressure ventilation Perfusion began 11 45 a.m.

- a 12 35 p.m. Ventilation with air  
b 12 37 p.m. Arrow and signal mark time of change to 5 per cent  $\text{CO}_2$  in air  
The increased volume of blood in the V.R. shows a decrease of L.B.V.  
c 12 47 p.m.  
d 1 06 p.m.  
e 1 16 p.m. Change to air at signal and arrow  
No change in P.A.p., which remained at 30 cm blood  
V.R. = venous reservoir

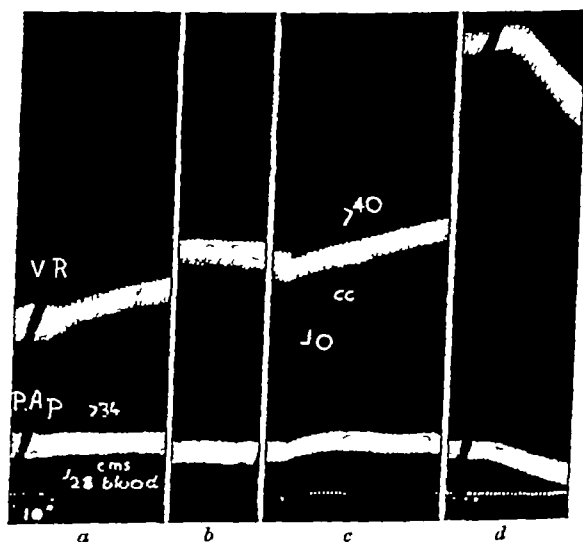


FIG 3—Expt 15 Dog, 12.8 kg Negative pressure ventilation Perfusion began at 11 30 a.m.

- a 12 32 p.m. Change from air to air containing 5 per cent  $\text{CO}_2$  (at signal) Tidal air 252 c.c.  
b 12 56 p.m. Tidal air 215 c.c.  
c 1 24 p.m. Change from air containing 5 per cent  $\text{CO}_2$  to air containing 10 per cent  $\text{CO}_2$  (at signal) Tidal air 200 c.c.  
d 1 55 p.m. Change from air containing 10 per cent  $\text{CO}_2$  to air (at signal) Tidal air 200 c.c.

TABLE III

No of Expt	Time from start of perfusion to test	Lung blood volume change c c from an arbitrary initial zero	Blood CO <sub>2</sub> content volume per cent at time of test
6	0-1 hr 45 min	+35	3.82
	2 hr 48 min	-13	33.01
	3 hr 38 min	+68	3.42
13	2 hr 36 min - 3 hr 25 min	+15	16-4.44
	4 hr 7 min	-32	28.93
15	0-1 hr 2 min	+35	8.42
	1 hr 50 min	-27	34.76
	2 hr 21 min	-55	62.03
16	0-0 hr 57 min	+18	12.02
	1 hr 2 min	-7	27.08
	1 hr 39 min	-17	34.62
	2 hr 2 min	-30	52.35
	2 hr 29 min	+49	21.09
17	0-0 hr 53 min	+33.5	8.25
	58 min	-6	28.33
	1 hr 34 min	-12.5	35.65
	1 hr 48 min	-27.5	51.74

gas analysis In the experiment of fig 6, the CO<sub>2</sub> response, which had been much reduced after dihydroergotamine, was again improved

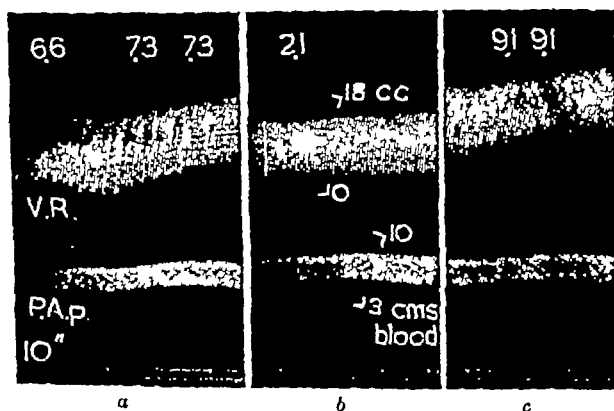


Fig 6—Expt 25 Dog, 15.0 kg Perfusion began at 11 03 a.m. Negative pressure ventilation

a 1 17 p.m.

b 2 00 p.m.

c 2 12 p.m.

Ventilation was with air, and a change made to 10 per cent CO<sub>2</sub> in air between the signals

2 mgm dihydroergotamine given between a and b at 1 47 p.m. (concentration in blood 1 225,000)

The lungs were inflated once by positive pressure between b and c

The top row of figures is the tidal air in c c

possible to calculate the volume of blood in the lungs at the beginning of perfusion, and for this reason it is not possible to express the changes in lung blood-volume as percentage changes of the total volume of blood in the lungs. However, in any one experiment the amount of blood in the lungs relative to an arbitrary initial value was inversely related to the  $\text{CO}_2$  content of the arterial blood (see fig 5 and Table III)

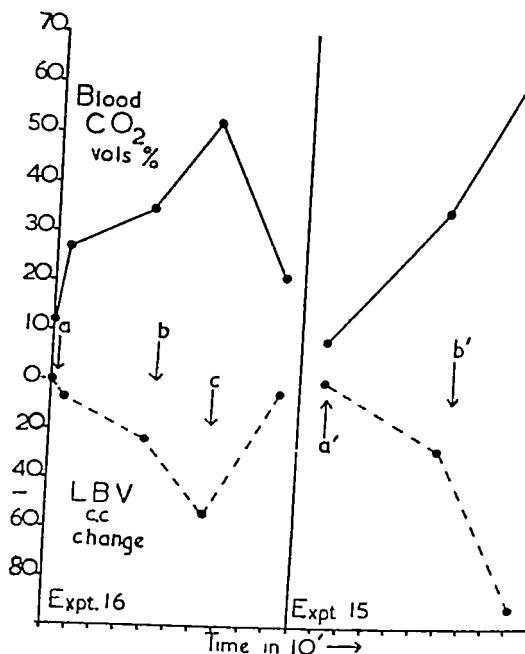


Fig 5—The inverse relationship between the  $\text{CO}_2$  content of the pulmonary arterial blood and the volume of blood in the lungs. The lung blood volume is plotted from an arbitrary zero. Two experiments are shown, both of which were carried out under negative pressure ventilation. The times of the changes of gas mixtures are shown thus:

- Expt 16 a Air to air containing 5 per cent  $\text{CO}_2$   
 b Air containing 5 per cent  $\text{CO}_2$  to air containing 10 per cent  $\text{CO}_2$   
 c Air containing 10 per cent  $\text{CO}_2$  to air  
 Expt 15 a Air to air containing 5 per cent  $\text{CO}_2$   
 b Air containing 5 per cent  $\text{CO}_2$  to air containing 10 per cent  $\text{CO}_2$

The response of the pulmonary blood-vessels to  $\text{CO}_2$  was not suppressed by ergotoxine ethane sulphonate (4 expts) or by dihydroergotamine (Sandoz) (2 expts). Both drugs were injected into the pulmonary arterial tubing to give blood concentrations (1:400,000–1:200,000) which were sufficient to reverse or to inhibit the pulmonary pressor response to adrenaline. The  $\text{CO}_2$  response was usually reduced after ergotoxine and ergotamine. Since both these drugs diminished the tidal air, it appeared likely that they also diminished the amount of  $\text{CO}_2$  admitted to the alveolus, a view which was supported by blood



obtained a reversal of the  $\text{CO}_2$  response in cat lungs after much larger doses of adrenaline

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The pulmonary vascular response to  $\text{CO}_2$  is not suppressed or reversed by either ergotoxine ethane sulphonate or dihydroergotamine, nor has atropine sulphate any effect This indicates that the effect is not likely to be mediated by adrenergic or cholinergic nervous elements, and that in all probability the effect is directly on the vessel wall

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when the tidal air was restored by inflating the lungs under positive pressure

Additions of atropine sulphate to the blood (conc 1 400,000-1 500,000) did not inhibit or otherwise affect the pulmonary vascular response to  $\text{CO}_2$  (3 expts)

*Response of the Pulmonary Vessels to Adrenaline during Ventilation of the Lungs with 5 per cent  $\text{CO}_2$  in Air*—Adrenaline was injected into the pulmonary artery in 13 experiments to see if variations in blood  $\text{CO}_2$  content had any effect on the response of the lung blood-vessels. The

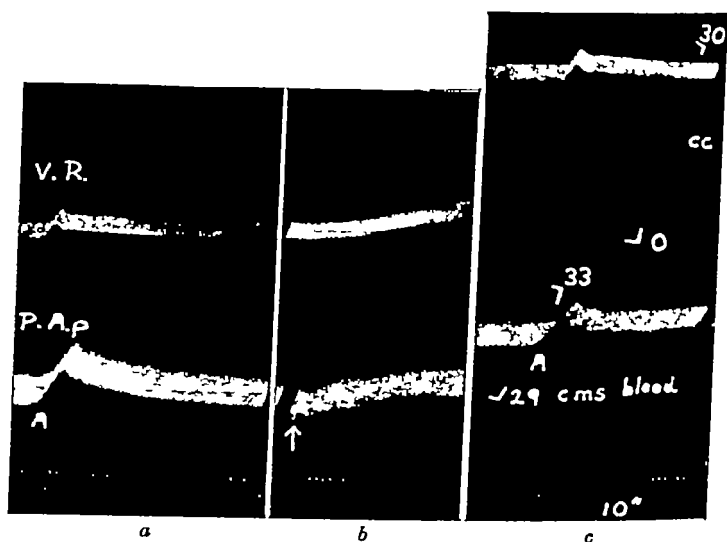


FIG 7—Expt 3 Dog, 120 kg 540 cc blood in apparatus Blood flow 310 cc/min. Perfusion began at 11 05 a.m. Positive pressure ventilation 10  $\mu\text{g}$  adrenaline was injected into the pulmonary artery every 10 minutes throughout the experiment

- a 2 30 p.m. Ventilation on air 10  $\mu\text{g}$  adrenaline at A
- b 2 43 p.m. Change to air containing 5 per cent  $\text{CO}_2$  at signal and arrow
- c 3 20 p.m. 37 minutes after changing to air containing 5 per cent  $\text{CO}_2$  10  $\mu\text{g}$  adrenaline at A

dose of adrenaline was kept constant in each experiment (5–20  $\mu\text{g}$  of Parke Davis' solution with chloretone) and tests were repeated at 10-minute intervals. The response was always the same—there was a transient rise in pulmonary arterial pressure and a coincident increase in the venous outflow. There was no difference in the response when air or air containing 5 per cent  $\text{CO}_2$  was used to ventilate the lungs, as shown by fig 7, in which 10  $\mu\text{g}$  adrenaline were injected into the pulmonary artery before and during administration of 5 per cent  $\text{CO}_2$  in air. Additions of adrenaline to the blood did not vary the usual pulmonary vascular response to 5 per cent  $\text{CO}_2$ , although Löhner [1924]

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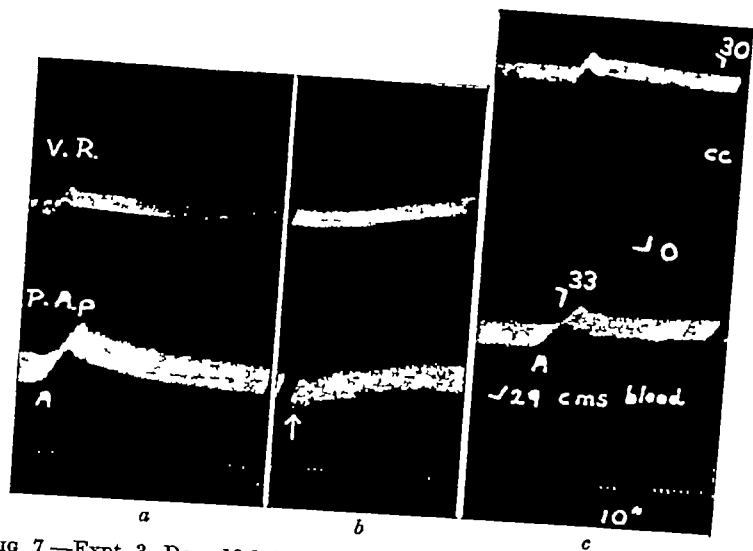


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blood-volume changes as have been observed could be accounted for by a capacity effect due to venous constriction alone, and the simplest explanation of the action of  $\text{CO}_2$  is that it causes constriction of the pulmonary capillaries, with or without concomitant constriction of the veins. It is also possible to visualize a more complex effect which would involve passive or active vasodilatation in some areas of the pulmonary vascular bed occurring simultaneously with a greater vasoconstriction elsewhere. In this event resistance effects might be minimized but the lung blood-volume changes would be large.

Isolated perfused lungs may be unsuitable preparations on which to test the effects of  $\text{CO}_2$ . In these experiments it was found that the blood in the pulmonary artery usually contained less  $\text{CO}_2$  and more  $\text{O}_2$  than mixed venous blood, and there can be no assurance that the pulmonary vessels of the normal lung, which are under very different conditions, will react to  $\text{CO}_2$  like those of the isolated lung. However, the results of Sjöstrand [1935] and Hochrein and Keller [1932] indicate that there is a diminution in the lung blood-volume of intact mice and dogs after inhalation of gas mixtures containing high concentrations of  $\text{CO}_2$ , and Von Euler and Liljestrand [1946] have found a pulmonary pressor response to  $\text{CO}_2$  in anesthetized cats.

### SUMMARY

1 Isolated dog lungs under negative or positive pressure ventilation have been perfused with blood through the pulmonary artery at constant volume inflow. The response of the pulmonary vessels to various concentrations of  $\text{CO}_2$  in the ventilating gas has been observed.

2 The capacity of the pulmonary blood-vessels is markedly diminished by increasing the concentration of  $\text{CO}_2$  inhaled from the small percentage present in atmospheric air to 5 or 10 per cent, and the pulmonary arterial pressure frequently shows a 5 to 20 per cent increase.

3 The lung blood-volume shows an inverse relationship to the  $\text{CO}_2$  content of the arterial blood.

4 The effect of adrenaline on the pulmonary vessels is not altered during ventilation of the lungs with 5 per cent  $\text{CO}_2$  in air.

5 The response to  $\text{CO}_2$  is not inhibited by ergotoxine or by atropine.

6 The possible site of action of  $\text{CO}_2$  is discussed.

### ACKNOWLEDGMENTS

I should like to thank Professor de Burgh Daly for much help and advice, and also Miss Catherine O. Hebb for assisting in the discussion of results.

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THE CHANGES IN PLASMA AND TISSUE FLUID VOLUME  
FOLLOWING EXERCISE By H CULLUMBINE and A C E  
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University of Ceylon, Colombo

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THERE is ample suggestive evidence that muscular exercise may produce marked changes in the distribution of water in the body. Barcroft and Kato [1915] demonstrated that there was an increase in the weight of skeletal muscle during activity. Fenn *et al* [see Fenn, 1936] have shown that there is an increase in the volume of extra-cellular fluid in the muscles of the rat and the frog following violent stimulation. Muscular exercise in man produces an increased concentration of the blood [Dill *et al*, 1930], while Gregersen [1941] has reported an experiment on one subject in whom the plasma volume (measured by means of the dye, T-1824) decreased by 16 per cent following exercise. To obtain a more precise estimate of the magnitude of these changes, simultaneous determinations of plasma volume and tissue fluid volume before and after exercise have been made.

### METHODS

Thirteen Ceylonese adult males were used as subjects. They rested for two hours before the commencement of the experiment and, except when exercising, they remained seated throughout the experiment. No fluid was taken by the subjects for 3 hours before the exercise and for at least 2 hours after the exercise. The exercise consisted of stepping up and stepping down from a stool 20 inches high and at a rate of 30 "step-up and step-down" cycles per minute. The exercise rate was maintained by means of a metronome and was performed for five and, in some cases, ten minutes. Such exercise can be considered to be moderate in severity, and Cogswell *et al* [1946] have shown that it produces a "steady state" in circulatory and respiratory responses.

In the first experiment, for which 6 subjects were used, the procedure was as follows —

After two hours' rest a sample of venous blood was withdrawn, and then about 10 c c (the actual volume varied a little from subject to subject but was carefully measured in each case) of a solution containing 50 mg Evans Blue dye and 500 mg sodium thiocyanate per 10 c c was injected intravenously. Venous blood samples were withdrawn

5, 10, 15, 20, 40, 60, 90 and 120 minutes later To minimise hæmolysis the blood samples were collected with paraffined syringes and needles and delivered into paraffin-coated centrifuge tubes containing 500 units Heparin (0.5 ml) The subjects exercised for five minutes immediately before the 40-minute blood sample was collected

Hæmatocrit readings were taken on the pre-injection, 20-, 40-, 60- and 90-minute samples Blood and plasma specific gravities were estimated by the copper sulphate technique of Phillips *et al* [1945] on all samples From these specific gravities the plasma protein concentration was determined

The concentrations of Evans Blue dye and of sodium thiocyanate in the plasma were determined for each sample The Evans Blue was estimated after extraction by the method of Crooke and Morris [1942] and the sodium thiocyanate by the method of Bowler [1944] Readings were made in a Spekker photoelectric colorimeter

The air temperature was 82.4° F and the relative humidity 78 per cent

### RESULTS

The "available fluid" volume was calculated by dividing the milligrams of thiocyanate injected by the milligrams of thiocyanate per litre of plasma [Gregersen and Stewart, 1939] This volume corresponds to "Space A" of Stewart and Rourke [1941] and Kaltreider *et al* [1941] "Interstitial fluid" volumes were calculated by subtracting the plasma volume and 70 per cent of the red cell volume from the available fluid volume

The relevant results are given diagrammatically in fig. 1, and the magnitude of the volume changes is indicated in Table I

TABLE I—VARIATIONS IN THE VOLUME OF THE BODY FLUIDS AFTER MODERATE EXERCISE

Subject	Volume in litres of					
	Plasma		Available fluid		Interstitial fluid	
	Before exercise	After exercise	Before exercise	After exercise	Before exercise	After exercise
1	2.75	2.25	14.78	16.97	11.04	13.77
2	2.83	2.40	11.54	13.49	7.78	10.20
3	2.20	1.60	13.59	14.82	10.75	12.63
4	2.83	2.03	15.32	17.61	11.59	14.47
5	2.05	1.60	10.20	11.81	7.52	9.48
6	2.28	2.05	14.78	16.95	11.78	14.16

The "plasma volumes" given here are calculated direct from the observed plasma concentrations of the Evans Blue dye, the assumption being made that the whole of the injected dye is still in the circulation

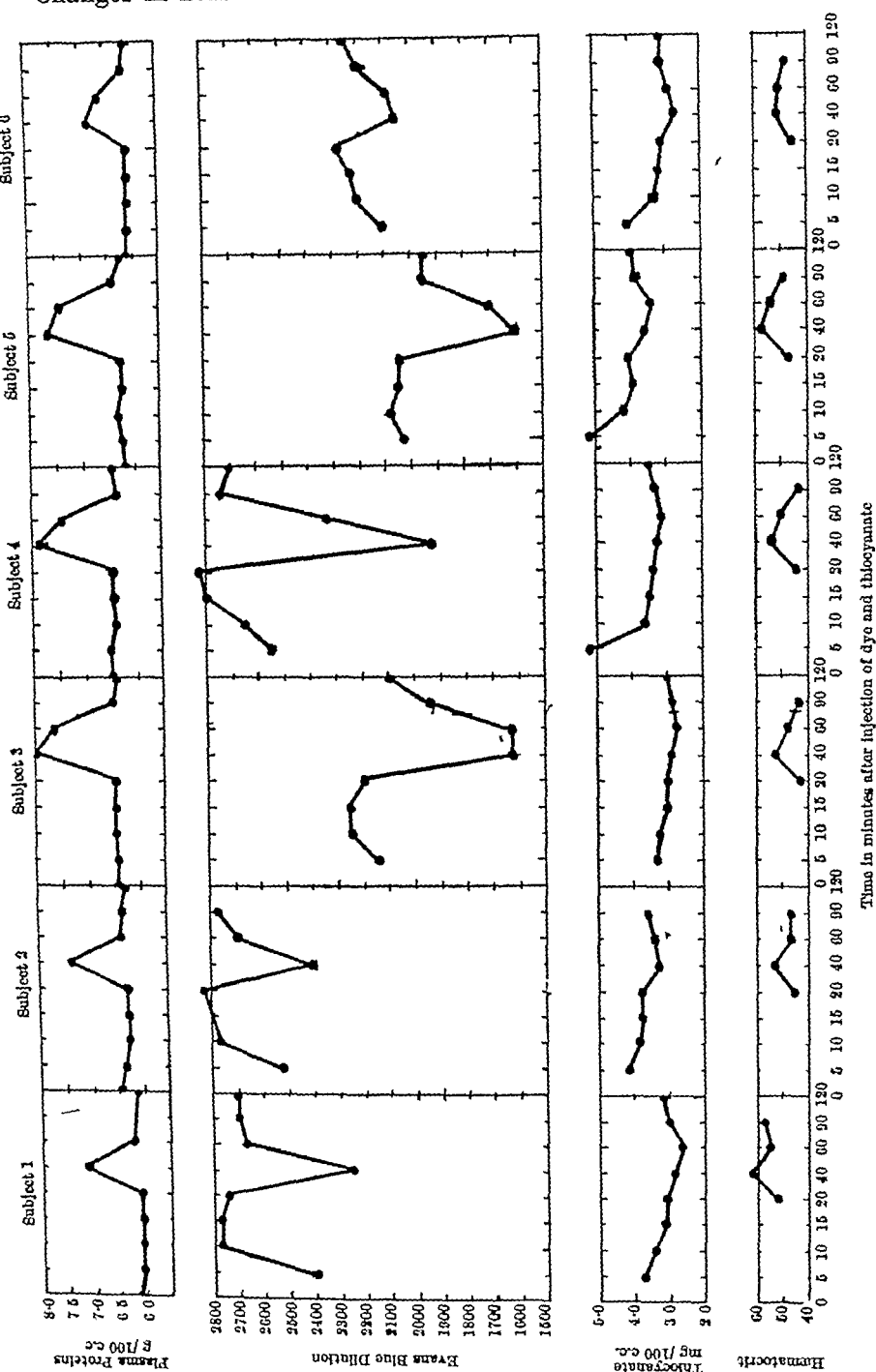


FIG. 1.—Variation in plasma protein concentration and body fluid volumes after 5 minutes' exercise, commencing at the 35th minute

It will be seen that moderate exercise of the nature of a 5-minute Harvard Step Test produces a hæmoconcentration, as evidenced by the rise in plasma protein content and the hæmatocrit readings

This hæmoconcentration is reflected in the fall in plasma volume, which is indicated by the changes in the plasma dilution of the Evans Blue dye. This fall in plasma volume varies between 225 to 800 c.c. in magnitude in the different subjects. The plasma volume does not return to normal for 40 to 60 minutes after the exercise.

There is also a fall in the concentration of plasma thiocyanate following the exercise. In each subject the plasma thiocyanate had reached a constant level before the exercise (i.e. the thiocyanate was uniformly distributed in the "available fluid" of the body), and the concentration returned to its constant level about 1 hour after the exercise. This fall in plasma thiocyanate concentration indicates an increase in the available fluid volume, and it will be seen from the table that the interstitial fluid volume also increases. Some increase in interstitial fluid volume would be expected, since the plasma lost from the circulation would have passed into the interstitial spaces. However, the increase in interstitial fluid volume is, in all cases, greater than can be accounted for by mere plasma diffusion. Presumably the water produced by the increased metabolism during muscular exercise has aided in this rise in interstitial fluid volume. In most cases too the maximum rise in available fluid volume occurs about 20 minutes later than the maximum hæmoconcentration.

To ensure that the variations in the plasma sodium thiocyanate and Evans Blue dye concentrations were caused by the exercise, the experiment was repeated on 4 of the 6 subjects, but this time no exercise was performed. In these instances no comparable increase in the concentration of Evans Blue dye and no fall in the concentration of sodium thiocyanate were found for three hours subsequent to the injection (see fig. 2).

The procedure adopted to demonstrate the change in plasma volume after exercise cannot give a measure of the absolute plasma volume after exercise, and, in addition, the return of the plasma volume to its normal pre-exercise level may be masked by the loss of dye from the circulation. To obtain a more accurate measure of the plasma volume immediately after exercise and to ascertain more precisely the time for return to normal, a different procedure was adopted.

Seven fresh subjects were used. A control sample of blood was obtained, and then the Evans Blue dye and the sodium thiocyanate injected intravenously. Blood samples were obtained 10, 20, 40, 60 and 90 minutes after this injection. When the 90-minute sample had been taken, the subjects at once performed the Harvard Test for 5 minutes. Immediately on completion of the exercise, 5 c.c. of 1 per cent Evans Blue dye solution was injected intravenously and further

venous blood samples obtained 10, 20, 40, 60 and 90 minutes after the exercise. All the blood samples were analysed as before and, in this way, an estimation of the plasma volume before and after exercise was

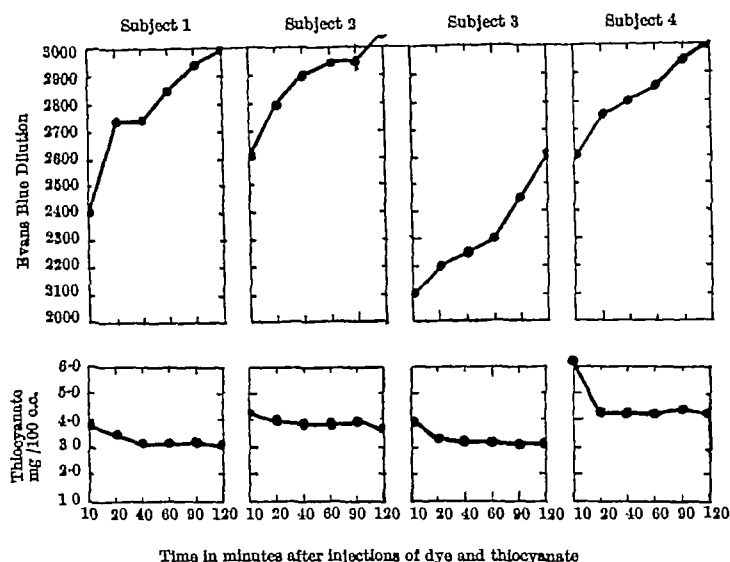


FIG. 2—Variations in body fluid volumes—resting subjects

obtained [Overbey *et al*, 1947, Cruickshank and Whitfield, 1945]. The air temperature was 84.3° F and the relative humidity 83 per cent. The results are summarised in Table II.

TABLE II—VARIATIONS IN THE VOLUME OF PLASMA AND AVAILABLE FLUID AFTER EXERCISE

Subject	Plasma volume in c c		Time to return to normal in minutes	Available fluid volume in litres		Time after exercise for maximum volume in minutes	Time to return to normal in minutes
	Before exercise	After exercise		Before exercise	After exercise		
7	2130	1840	25	11.446	12.05	10	40
8	2250	1900	25	13.163	13.900	30	50
9	2450	2050	25	13.163	14.040	40	60
10	2850	2300	30	13.320	14.450	40	60
11	3100	2600	75	14.70	15.85	60	90
12	2950	2700	75	12.40	13.16	60	90
13	2450	1900	60	10.88	11.88	60	90

Again it is obvious that the plasma volume decreases and the available fluid volume increases after exercise

Subjects 11, 12 and 13 performed the Harvard Test for 10 minutes. The fluid volume changes are not noticeably more with the longer exercise, though the individual variation is too great and the number of subjects too small to justify a precise statement. However, after the longer exercise the available fluid does seem to increase for a longer time than with the shorter exercise. In all cases the maximum available fluid volume occurred later than the maximum fall in plasma volume, and, indeed, in some cases the plasma volume had returned to normal while the total available fluid was still increasing in quantity.

### DISCUSSION

As suggested by the results of earlier workers [Gregersen, 1941, Dill *et al*, 1930], exercise results in a decrease in the plasma volume. In our experiments there were wide individual variations in the amount of fluid leaving the blood-stream (225 to 800 c c), but in all cases the loss of fluid occurred rapidly, and this loss was restored within 20 to 30 minutes for exercise of 5 minutes' duration and within about 75 minutes for exercise lasting 10 minutes.

Even greater changes occurred in the volume of "available" and "interstitial" fluids. Here, too, wide individual variations in response occurred (*e g* 0.604 to 2.29 litres was the range of increase in available fluid volume). The maximum increase in the available and interstitial fluid volume in 11 of the 13 subjects occurred 20 to 60 minutes after the cessation of the exercise and the occurrence of maximum hæmo-concentration. The longer the duration of exercise, the longer was the persistence of these increased available and interstitial fluid volumes. These fluid volumes were calculated after estimating the plasma concentration of sodium thiocyanate, and the changes in the concentration of this substance produced by exercise could be interpreted in other ways. Thus exercise may have induced a greater rate of excretion of sodium thiocyanate in the urine. If this had occurred, then we should not expect the plasma concentration to return, as it did, fairly rapidly to its pre-exercise level. Or it may be suggested that, during exercise, part of the sodium thiocyanate is removed from the body fluids and becomes an intracellular constituent. After exercise this intracellular sodium thiocyanate would have to be released to restore the plasma concentration. In the absence, at present, of any evidence to support this, we must assume that the changes in plasma sodium thiocyanate concentration following exercise reflect changes in the volumes of the body fluids.

Estimations of the total body water before and after exercise would help in the interpretation of our results. Painter [1940] has used urea and sulphanilamide for estimating the total body water in dogs. Their use in man would be complicated by the alteration in the rate of excretion of urea by the kidney during exercise, and the fact that sulphanilamide is conjugated in the liver of man but not in dogs. The Appendix gives details of age, height and weight of the 13 subjects.

## APPENDIX

## AGE, HEIGHT AND WEIGHT OF THE SUBJECTS EXAMINED

Subjects	Age, years	Height, cm	Weight, lb
11	21	179	148
12	22	173	143
13	23	150	94
10	22	162	151
3	25	154	101
7	31	153	89
2	19	156	92
5	22	157	90
6	24	165	118
4	21	154	113
1	46	158	140
9	36	156	125
8	24	153	98

## SUMMARY

Simultaneous determinations of plasma volume and tissue fluid volume before and after exercise have been made in 13 male Ceylonese. Exercise produces a prompt decrease in plasma volume, and a slower but greater increase in available fluid and interstitial fluid volume.

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COMMUNICATING BLOOD VESSELS BETWEEN BRONCHIAL  
AND PULMONARY CIRCULATIONS IN THE GUINEA-  
PIG AND RAT<sup>1</sup> By PRABHASCHANDRA RAKSHIT From  
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THE bronchial arteries originally described by Marchetti [1664] and later by Reisseissen [1822] and Luschka [1863] have been examined in a number of animal species [Miller, 1906, 1907, 1919 b, 1937] For the most part they arise from the aorta and intercostal arteries, and supply all the tissues of the lung and the respiratory tree as far as the respiratory bronchioles There is some variation in the blood-supply of the *pleura pulmonalis* in different animal species, for it is supplied by the bronchial arteries in man, the sheep and the horse, but in the dog the pulmonary arteries are the chief source of supply [Miller, 1937]

It is generally accepted that the capillaries of the bronchial vascular system in the region of the respiratory bronchioles communicate with those of the pulmonary circulation The drainage of blood from the 1st, 2nd and 3rd order of bronchi is by way of the bronchial veins and from the more distal part of the respiratory tree by the broncho-pulmonary veins [Miller, 1937, Berry and Daly, 1931, Daly, 1938] The bulk of evidence is against the existence of communicating vessels between the bronchial and pulmonary arteries [Berry, Brailsford and Daly, 1931, Daly, 1936]

Under certain pathological conditions it has been found that the bronchial arteries may increase in size and in number and also establish a greater communication with the pulmonary circulation than normally [Guillot, 1845, Virchow, 1847, 1851, 1856, Küttner, 1874, 1878, Guyot, 1906, Christeller, 1916, Wood, Crever and Miller, 1937, Karsner and Ash, 1912, Karsner and Ghoyreb, 1913, Schlaepfer, 1923, Holman and Mathes, 1929] Under these conditions it is possible that bronchial arterial vessels may open directly into pulmonary arterial branches It was observed, however, by Daly [1938] that in apparently healthy guinea-pigs killed by a neck-blow, the appearance in the pulmonary vascular bed of material injected into the aorta was more rapid than if

<sup>1</sup> Observations collated from a thesis presented for the Ph D degree of the University of Edinburgh, January 1940

injected into the pulmonary artery itself. The phenomenon was so striking that he suggested it might be accounted for by pulmonary vasospasm due to the procedure adopted for killing, and by the existence of communicating vessels between the bronchial and pulmonary arteries. The phenomenon had not been observed in similar experiments on dog lungs.

The aim of the experiments to be described was to discover whether in healthy guinea-pigs and rats bronchial arterial and pulmonary arterial communicating vessels could be demonstrated.

### METHODS

(1) Injection of a celloidin mass (3-7 per cent followed by a 7-10 per cent solution) coloured with alkalin (Schering) into the pulmonary artery, aorta and pulmonary veins, with subsequent digestion of the soft tissues with hydrochloric acid and examination of the casts with the aid of a dissecting microscope. The celloidin was prepared according to the method of Hinman, Morrison and Brown [1923].

(2) Injection of (a) carmine gelatine (12.5 per cent), (b) Nicholson's blue gelatine (10 per cent), or (c) Prussian blue glycerine mass (Beale). Their preparation is described by Rakshit [1940]. They were injected into one or into both arterial systems. Frozen and paraffin sections of the injected lungs were then prepared and serial sections examined under the microscope.

In both methods the animals were killed by a blow on the neck and then placed on a warm tray. The thorax was opened and the roots of the pulmonary artery and aorta defined by blunt dissection. Loose ligatures were placed around these vessels. The main branches of the aorta and the descending parts of the aorta below the level of the lung hilus were ligated. Cannulae were inserted into the aorta and pulmonary artery. The right auricle was opened. In the majority of animals warm saline was run through the aorta alone until the effluent from the right auricle was clear. In the remaining experiments saline was also run through the pulmonary artery until the effluent from the left auricle was clear.

The injection material was kept in warm pressure bottles placed at the head of the animal. In a few cases the celloidin mass was injected into the respiratory tree and into the pulmonary veins (via the left auricle) separately or in addition to the arterial injections. The average pressures of injection were into the pulmonary artery, 50 mm Hg, pulmonary veins, 35 mm Hg, aorta, 150 mm Hg, and trachea, 35 mm Hg.

### RESULTS

*Guinea-pigs*—Figs 1-3 are photographs of casts obtained by injection of celloidin. The casts of the pulmonary artery and of the



FIG 1



FIG 2



FIG 3



FIG 5



FIG 6

- FIG 1 —Guinea pig Celloidin casts of portion of respiratory tree (1) and of pulmonary artery (2)
- FIG 2 —Guinea pig Celloidin casts of portion of respiratory tree (1) and of bronchial arteries (2, 3, 4)
- FIG 3 —Guinea pig Portion of celloidin casts of bronchial artery (1), pulmonary artery (2) and aorta (5) Esophagus = (6) For description see text
- FIG 5 —Rat. Celloidin casts of pulmonary artery (1) and pulmonary vein (2)
- FIG 6 —Rat Celloidin casts of aorta and branches (1), pulmonary artery (2) and respiratory tree (3) For description see text.

respiratory tree (fig 1) show a smooth outline. Casts as a result of injecting the aorta and respiratory tree (fig 2) did not always show a close approximation of the bronchial arteries to the bronchi, which is striking in other animal species. This is due, at any rate in part, to the mechanical displacement of the delicate bronchial arterial cast from the stouter bronchial cast. Even when the greatest care is taken in the final washing, the absence of a continuous support of the arterial cast by the bronchial cast leads to some displacement. The thicker swellings on the arterial cast are apparently due to the filling of adjacent air sac vessels, some of which may have been ruptured.

The portion of the cast shown in fig 3 was obtained after simultaneous injection of the aorta (red) and pulmonary artery (blue). The red injection material appeared in the cavity of the left auricle. The dark wavy line between 3 and 4 marked on the photograph is a small vessel lying on a pulmonary artery. At its upper end (4) it is blue, in its mid-portion purple, and at the lower end (3) red. The upper portion of this small artery (blue) is attached smoothly to the pulmonary artery cast, and the lower end (red) can be traced from an undoubted bronchial artery. The diameter of the artery is  $90\ \mu$  and it is probably too large to be a *vas vasorum*. Further, it is unlikely that the injection mass would find its way from the lumen of the pulmonary artery into the ramifications of a *vas vasorum*, and so into the main *vas vasorum*, unless rupture of the larger ramifications into the cavity of the pulmonary artery had occurred. The smoothness of the junction of the small vessel with the main pulmonary artery branch suggested that no such rupture had occurred.

In another cast a similar relation between a bronchial artery and pulmonary artery was observed. In this case a much longer length of the bronchial artery where it joined the pulmonary artery (blue) was coloured blue. This suggests that during the simultaneous injection of the aorta and pulmonary artery the injection mass had passed without difficulty from the pulmonary artery into the bronchial artery.

Aortic injections of carmine gelatine, or of carmine gelatine containing starch granules for the purpose of blocking the capillaries, readily found their way into the bronchial arteries supplying the bronchial walls, the lymphatic glands and the *vasa vasorum* (fig 4). The pulmonary arteries and veins were also readily filled with the injection mass.

*Rat*—Fig 5 shows casts of the pulmonary artery and vein, and fig 6 casts obtained by injecting the aorta (red), pulmonary artery (blue) and trachea (white). In the last specimen injection into the pulmonary artery was started  $2\frac{1}{2}$  hours after the aortic injection. Just above the numeral 5 there is a small looping artery nearly horizontal and  $75\ \mu$  in diameter. One end of the loop is continuous with a bronchial artery and is red in colour. The other end of the loop is blue, and passes towards the bifurcation of the pulmonary artery to be continued down

the primary bronchus, where it becomes red in colour At this point it gives off a branch which enters the pulmonary artery The mixing of



FIG 4—Guinea-pig Carmine gelatine injected into the aorta B=bronchus, P=pulmonary vein The arteries supplying the walls of the bronchi and the larger pulmonary blood vessels are filled with carmine which shows up black in the photograph Some of the capillaries in this specimen are also filled with carmine gelatine

the red and blue injection masses in this branch again suggests that it is a communicating branch between the bronchial arteries and pulmonary artery A fine bronchial arterial twig supplying the bronchus is seen running horizontally just to the left of 6



FIG 7—Rat Bronchial arteries (black) in the walls of bronchus, the pulmonary artery, and in a lymph node

Aortic injections of barium sulphate filled the arteries to the bronchi and lymph glands without injecting the pulmonary capillaries (fig 7). Also barium sulphate injections into the pulmonary circulation and carmine gelatine into the aorta led to red injection material reaching the arteries to the bronchi and lymph glands but not the pulmonary blood-vessels. These results indicate that the bronchi and lymph glands are supplied by the bronchial arteries.

### DISCUSSION AND CONCLUSIONS

The ease with which injection masses into the aorta reach the pulmonary circulation in the guinea-pig is confirmed. This militates against a clear-cut separation of the bronchial and pulmonary circulations by the injection-mass method in this species of animal. Attempts to confine the injection mass to one circulation by using a coarse injection mass (starch granules) were not successful, but in specimens in which the mixing of the two injection masses was restricted to the pulmonary veins and a few capillaries, only the aortic injection material filled the arterial supply to the larger bronchi (including the vessels to the bronchial mucosa), the *vasa vasorum* of the larger pulmonary arteries, the bronchial musculature and cartilage, and the interstitial tissue of the lungs. There appears therefore strong presumptive evidence that these tissues normally receive their blood-supply from the bronchial arteries.

The two vessels connecting the bronchial and pulmonary arteries of which a description is given in detail, one in the guinea-pig, the other in the rat, were smooth in contour. The absence of raggedness at their point of junction with the pulmonary artery, as well as their size, militate against the view that they are *vasa vasorum*. Blood-vessels of a similar nature were seen which had a broken end, and were red and blue in colour at each end and purple in their middle portion. These as well as the two communicating vessels described, were in the region of the lung hilus and ranged from 70 to 95  $\mu$  in diameter.

If, as it is believed, these vessels are communicating channels between bronchial and pulmonary arteries, it would account for the easy passage of aortic injection material into the pulmonary circulation, but we have no direct evidence that this is the correct explanation.

### SUMMARY

- 1 In the guinea-pig, the ease with which injection masses into the aorta find their way into the pulmonary circulation has been confirmed.
- 2 Evidence is presented which indicates that in the guinea pig and rat the bronchial arteries supply the bronchi, the lymph glands and the interstitial tissue of the lungs. They also give rise to the *vasa vasorum*.

3 In the guinea-pig and rat, casts of vessels connecting the bronchial artery and pulmonary artery casts are found in the region of the lung hilus. Reasons are given for the belief that they represent communicating vessels and not *vasa vasorum*.

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# SOME OBSERVATIONS ON THE COMPARATIVE EFFECTS OF COLD AND BURNS ON PROTEIN METABOLISM IN RATS

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(Received for publication 18th February 1940)

IN recent years considerable attention has been devoted to the metabolic disturbances which are set up by various types of trauma Cuthbertson [1932] especially has shown that an increased excretion of nitrogen is one of the most striking expressions of this altered metabolism

In studies of protein metabolism in burned rats in 1945, it was noted by Peters in a previously unpublished experiment (experiment 1 of the series now reported) that when the environmental temperature fell during a period of cold weather, the nitrogen excretion of the burned animals was not additionally increased, suggesting some initial common path In contrast, control animals excreted nitrogen in amounts which approached the increased excretion in the burned animals This observation has led us to perform some experiments upon the relation between environmental temperature, burns and nitrogen excretion Since cold is frequently considered to stimulate the thyroid, this has also been investigated in parallel researches [Gribble and Peters, 1949] Since also the stimulus to some of this work has been the observations by Croft and Peters [1945] upon the effect of methionine, the influence of this upon the increases in nitrogen excretion under cool conditions has also been examined Though not complete, it has seemed advisable to report our experiments for the benefit of others working in the field, as we are unable to continue them

## EXPERIMENTAL METHODS

The methods used in experiment 1 (1945) were adapted from those previously reported from this laboratory by Croft and Peters [1945] Some changes were made in experiments 2, 3 and 4, in which albino rats were maintained in individual cages placed over glass bowls containing a perforated zinc plate for the separation of faeces from the urine, which passed through into 50 ml of 0.1 N  $\text{H}_2\text{SO}_4$  Urine was

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removed at intervals, as stated in the individual experiments, made up to a fixed volume, and the ammonia and urea nitrogen determined as previously described. Throughout this paper nitrogen excretion refers to ammonia and urea nitrogen only. It has been assumed that the faecal N remains practically constant as was found by Croft and Peters [1945].

*Feeding*—Experiment I was made by the previous technique, with the addition of paired feeding to control the lowered food intake through failure of appetite after burning.

In the experiments of 1946 (2, 3 and 4 of the present paper), in order to circumvent the difficulties and disadvantages of the paired feeding technique, all animals were tube-fed with a diet of the following composition —

		Percentage by weight of calorie components	Percentage of calories
Casein (Glaxo A.E.)	33 g	17.7	13.0
Arachis oil	42.2 ml	24.8	41.2
Cod liver oil	4.0 ml		
Wheat starch	107 g	57.4	45
Salt mixture	3 g		
Agar	3.8 g		
Vitamin supplement	6 ml		
Water	176 ml		
Total volume	336 ml		

This diet was modified from the 14 per cent protein diet previously used in this laboratory. These modifications consisted of (1) replacement of casein and yeast by casein alone, (2) substitution of a portion of the carbohydrate by arachis oil, and (3) the use of purified vitamin preparations. It was thought that in these short experiments the animals would not run out of other unknown B factors. The diet was homogenized in a Waring blender, ice-cold water being used to prevent the starch from swelling. The diet was administered by tube twice daily, at nine in the morning and five in the afternoon, a No. 3 hard rubber catheter attached to a 10-ml metal-glass syringe being used [Lathe, 1948]. The animals were held over the cages during feeding in order to prevent urine loss, and weighed immediately thereafter, allowance being made for the weight of food administered. All the animals were fasted for 16 hours before the initial tube feeding. Water was available to the animals at all times.

Fifteen ml of the diet provided 18 cals per 100 g for a 240 g rat. It contained 235 mg of nitrogen. The vitamin supplement

consisted of aneurin, 10 mg, riboflavin, 10 mg, choline chloride, 6 g, Ca *d*-pantothenate, 30 mg, nicotinic acid amide, 20 mg, *p* aminobenzoic acid, 30 mg, pyridoxine hydrochloride, 7 mg, inositol, 2 g, 10 per cent ethanol, to 60 ml. When a methionine supplement was given, it consisted of 100 mg of DL-methionine incorporated in the diet.

Except where otherwise stated, the animals were kept in a constant temperature room at 22° C. Where lower temperatures were desired, these were obtained by exposing the animals in an open room during the autumn of the year. Under these circumstances the temperature depended on the weather. A recording thermometer was used in each such experiment to indicate the degree of cold to which the animals had been subjected. Even a drop of 2° C is sufficient to change the metabolism of rats on a constant diet.

Burning was carried out as previously described [Croft and Peters, 1945] at 73° C for 30 seconds. Ether was used to anaesthetize burned and control rats. All the experiments consisted of a preliminary basal period during which the nitrogen excretion on the diet was estimated. Then followed an experimental period of several days, which was begun by burning the animals, or placing them in the cold. Comparisons are made of the average daily nitrogen excretion during the basal and the experimental periods, and between the nitrogen excretion of different groups of animals.

## RESULTS

In experiment 1, which was made in 1945 with the technical assistance of R. W. Wakelin and which led to the further work, the N excretion of 12 adult rats was studied, these were divided into two groups of 6. They were fed on the basal diet of Croft and Peters [1945], supplemented with choline hydrochloride (10 mg per 100 g), after the preliminary period upon this diet 6 of the animals were anaesthetized and burned and the remainder anaesthetized and used as controls. It happened that during the latter course of the experiment, the simultaneous presence of a cold spell and of a failure in the heating of the animal house led to a fall in environmental temperature to which the animals were exposed. The average excretions per rat per diem are given in Table I for the basal period and for the 7 days following burning (excluding the first day).

The increase in the excretion of the burned animals fell within that to be expected in this series (+530 mg for 9 days), on the other hand, the control animals showed an increased excretion little different and falling within that previously observed for burning (+420 mg for 9 days). This suggested that the N excretion due to cold was not additive to that of the burn.

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**Feeding**—Experiment 1 was made by the previous technique, with the addition of paired feeding to control the lowered food intake through failure of appetite after burning

In the experiments of 1946 (2, 3 and 4 of the present paper), in order to circumvent the difficulties and disadvantages of the paired feeding technique, all animals were tube-fed with a diet of the following composition —

Percentage of calories	Percentage by weight of calorie components	Total volume	
		Casein (Glaxo A E)	Water
13.9	17.7	33 g	176 ml
41.2	24.8	Arachis oil (4.2 ml) Cod liver oil (4.0 ml)	6 ml
46	57.4	Wheat starch Salt mixture Agar Vitamin supplement	3.8 g 3 g 3.8 g 336 ml

This diet was modified from the 14 per cent protein diet previously used in this laboratory. These modifications consisted of (1) replacement of casein and yeast by casein alone, (2) substitution of a portion of the carbohydrate by arachis oil, and (3) the use of purified vitamin preparations. It was thought that in these short experiments the animals would not run out of other unknown B factors. The diet was homogenized in a Waring blender, ice-cold water being used to prevent the starch from swelling. The diet was administered by tube twice daily, at nine in the morning and five in the afternoon, a No. 3 hard rubber catheter attached to a 10-ml metal-glass syringe being used [Lathé, 1948]. The animals were held over the cages during feeding in order to prevent urine loss, and weighed immediately thereafter, allowance being made for the weight of food administered. All the animals were fasted for 16 hours before the initial tube feeding. Water was available to the animals at all times.

Fifteen ml of the diet provided 18 cal per 100 g for a 240-g rat. It contained 235 mg of nitrogen. The vitamin supplement

TABLE I—CHANGE IN EXCRETION OF N (MG PER DIEM PER RAT) OF CONTROLS, DUE TO COLD ENVIRONMENT, IN A BURNING EXPERIMENT (EXP 1)

	Mean excretion of N (mg /diem/rat)		Difference between means
	Before burning (5 days)	After burning (7 days)	
Control	175 1	221 7	+46 6±6 82
Burned	181 2	240	+58 8±12 23

In all tables the figures following  $\pm$  are the standard error of the mean, or difference between means, as calculated from individual figures

### *Effects of Cold and of Methionine*

In the 1946 experiments in which 51 rats were used, it was important at the outset to obtain some quantitative estimate of the effect of cold on nitrogen excretion, as well as any possible influence of methionine. In experiment 2, 19 rats were kept in the constant temperature room for a basal period of 2 days, during which the nitrogen excretion was estimated daily. They were then divided into four groups and treated as follows: group 1 remained under identical conditions, group 2 received a daily supplement of 100 mg of DL-methionine in the food, group 3 was transferred to the cold room where the temperature varied from 10° to 16° C, and group 4 was transferred to the cold room and received the methionine supplement. The experimental period lasted 7 days. The initial weight of the animals varied from 208 to 307 g, averaging 256 g. Each animal received 16 ml of diet per day (equivalent to 18 calories per 100 g of body-weight for a rat of 256 g). The summarized effect of cold, both with and without methionine, is indicated in Table II. The individual nitrogen excretion and weight changes are given in Appendix Table V.

Experiment 2 shows that, as would be expected on a constant diet, cold increases the daily nitrogen excretion, and that a methionine supplement has little if any effect upon this. Examination of the detailed results (Appendix Table V) indicates that the cold stimulus in 3/5 animals caused a rise in N excretion on the first day, in 4/5 upon the second day, and in all animals by the third day.

### *Effects of Cold and of Burning*

Experiment 3 was designed to determine whether the effects of cold and of burning were additive. Thirteen rats were divided into three groups, which were maintained during a four-day basal period in the

TABLE II —COMPARISON OF N EXCRETION OF GROUPS OF RATS MAINTAINED UNDER WARM AND COLD (15° C) ENVIRONMENTAL CONDITIONS WITH AND WITHOUT ADDITION OF METHIONINE (EXP 2)

	Average daily nitrogen excretion (mg)			Average weight change (g)	
	Number of animals	Preliminary period	Experimental period	Preliminary period	Experimental period
In warm	5	203	173	- 4	- 3
„ „ +meth	4	219	192	- 7	- 3
In cold	5	224	250	- 10	- 27
„ „ +meth	4	214	237	- 8	- 26
		Change in average daily N excretion (mg)			
		Warm	Cold	Effect of cold	
Standard diet		- 30 ± 8.4	+ 26 ± 4.5	+ 56	
+Methionine supplement		- 27 ± 6.4	+ 23 ± 2.9	+ 50	

constant temperature room. Initial animal weights varied from 246 to 266 g, and averaged 257 g. At the conclusion of the basal period two groups, 1 and 2, were burned (day 5), all animals were anaesthetized, and the following day groups 1 and 3 were placed in the cold room, where the temperature varied between 15° and 20° C. The experiment concluded on day 11. There was a slight difference between groups 1 and 2 and group 3. The latter served as a control for another experiment and was fed a slightly higher diet. Groups 1 and 2 received 17 calories per 100 g of body-weight, and group 3 received 18 calories per 100 g. The changes in mean daily nitrogen excretion and in weight, between basal and experimental periods for the three groups, are given in Table III. Nitrogen excretion of individuals is given in Appendix Table VI.

In experiment 3 the mean daily change in the cold was of the same order as in experiment 1 (+ 48). The burned animals kept in the warm excreted + 89 mg extra N, those in the cold + 78 mg. This is so much less than the combined figure of 137 mg that it confirms the impression given in experiment 1 that the stimuli for the burn and for cold in this

TABLE III—COMPARISON OF N EXCRETION FOR ANIMALS BURNED AND KEPT UNDER WARM AND COOL (17° C) ENVIRONMENTAL CONDITIONS (EXP 3)

Group	Number of animals	Treatment	Mean daily excretion (mg /rat/diem)		Difference due to treatment
			Basal period	Experimental period	
1	4	Cold after burning	184	202	+78 ± 20
2	4	Warm after burning	186	275	+89 ± 9 2
3	5	Not burned In cold	190	238	+48 ± 18 1

Weight changes Group 1, -24 g, 2, -14 g, 3, -12 g

experiment drew upon some source of N by a common path, 3/4 animals showed the increased excretion in the cold by the first day and 4/5 by the second day

#### *Effects of Cold, Burning and Methionine*

In experiment 4, cold, burning and methionine were compared simultaneously. Nineteen animals, averaging 276 g, were maintained for a basal period of 5 days at 22° C. They received 19 calories per 100 g. The diet was of the same composition as previously, except that the inositol in the vitamin supplement was reduced to 1/4 of the previous level. During the experimental period the animals were divided into four groups as follows: group 1 was handled as during the basal period, group 2 was burned, group 3 was given a daily supplement of 100 mg of DL-methionine in the diet, and group 4 was burned and received the methionine supplement. All animals were anesthetized. On the second day of the experimental period all animals were transferred to the cold room, where the temperature fluctuated from 6° to 16° C. The differences between the mean daily nitrogen excretion during basal and experimental periods is given in Table IV.

It will be seen that in experiment 4 the cold induced somewhat of the same change as before. However, the combined effect of cold and burning showed an additional increase which was statistically significant. It is a pity that, owing to technical difficulties, an extra control group in the warm could not be included, but the values for burning and keeping in the warm do not often much exceed +80 mg, and for a 9-day period are usually lower. In experiment 4 the experimental temperature was lower than in the other experiments. Methionine



TABLE IV—COMPARISON OF EFFECTS OF COLD (11° C), BURNING AND METHIONINE UPON N EXCRETION (EXP 4)

Group	Number of animals	Mean N excretion (mg /diem/rat)			Change
		Treatment for experimental period	Basal period (5 days)	Experimental period (6 days)	
1	5	Cold	186	251	65 ± 7.45
2	4	Cold + burn	181	307	126 ± 11.1
3	5	Cold + methionine	181	264	83 ± 16.7
4	5	Cold + burn + methionine	180	313	133 ± 16

## DISCUSSION

That under certain conditions cold [Lusk, 1929] and burns individually will increase nitrogen excretion in animals is now well established [Clark, Peters and Rossiter, 1945]. In any comparison of their effects two questions would seem to be of paramount importance (1) Are the effects on N excretion of approximately the same order, and are they additive? (2) Does the time from stimulus to response vary from one type of stimulus to another?

(1) In experiments 1, 3 and 4 the average excess nitrogen excreted as a result of burning is seen to be somewhat greater than that from cold. The effect of the cold alone varied from 48 to 65 mg, while that of burning and cold was 59 mg in experiment 1, 78 mg in experiment 3 and 126 mg in experiment 4. Hence in regard to the question whether the effects of cold and of burning are additive, an unequivocal answer has not been obtained. In experiment 3 the combined effect of cold and of burning was slightly less than that of burning alone. This is consistent with experiment 1, and shows that there may be no additive effect. However, in experiment 4, burning plus cold showed an apparent increase over and above the effect of the cold, this may be due to the greater degree of cold, but it shows that the matter is not quite straightforward. Nevertheless, there is a suggestion that a common path is partly involved.

(2) The speed of response to the stimulus of cold or of burning is important in relation to possible thyroid effects. In our experiments 1 and 2 the effect of cold on the N excretion was often present in 24 hours and usually marked in 48 hours. The excess N excretion as a result of burns may appear within 24 hours [Croft and Peters, 1945, confirmed

experiment 2] and reach a peak in 3-7 days. A similar time element is found in the N excretion following fractures in the rat [Cuthbertson, 1939] and following skeletal trauma in the human [Cuthbertson, 1932]. In the absence of a control of the degree of cold, it is impossible to say whether this is a qualitative or quantitative difference.

In regard to the effects of methionine, if account is taken of the extra 11 mg N added in the diet in the methionine supplement, experiment 2 or 4 shows that this addition neither prevented the N loss due to cold, nor that due to cold and burning. Gribble *et al* [1949] have discussed the problems of methionine and burns. The effects of excess of calories given as sugar in burning are discussed in a separate paper [Lathe and Peters, 1949], as also the possible influence of thyroid factors [Gribble and Peters, 1949]. It is evident that, even under ideal conditions, a constant nitrogen excretion in experiments involving a constant calorie intake can only be obtained when the temperature is rigidly controlled. Failing this, the increased excretion of controls under cool conditions might lead to the erroneous conclusion that cold reduces the N loss. The difficulties of experiments in this field are well illustrated by Appendix Tables V and VI, giving the detailed results of experiments 2 and 3. Even when constant amounts of nitrogen are introduced into the animal each day, the daily variations in excretion of individual rats are considerable. To reduce these to statistical order much larger numbers of animals would be required.

### SUMMARY

1 The comparative effects on nitrogen excretion of cold and burns have been investigated in preliminary experiments by a study of nitrogen excretion in rats receiving a constant calorie and protein intake on a high fat diet administered by tube.

2 Under these conditions, as is to be expected, cold and burns produce an increase in nitrogen excretion within 24 or 48 hours.

3 In two experiments the effects of cold and of burning on nitrogen excretion were not additive, in one at a colder temperature N loss was increased by burning.

4 A methionine supplement does not reduce the excess nitrogen excretion due to cold, or the excess nitrogen excretion due to burning in the cold.

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APPENDIX TABLE V—INDIVIDUAL VALUES FOR EXPERIMENT 2

Nitrogen excretion (mg)									
Days	1	2	3	4	5	6	7	8	9
In warm									
	206	260	177	201	209	197*	197*	184	177
	225	208	204	210	201	190	190	172	240
	237	246	221	207	203	187	187	157	178
	177	152	144	137	122	110	110	133	122
	170	149	155	170	160	155	155	138	137
Mean	203	203	180	185	179	168	168	157	171
In warm + methionine									
	241	243	215	208	191	196	196	177	197
	173	155	147	141	135	125	125	127	112
	221	207	225	206	209	178	178	190	212
	255	259	252	259	232	238	238	235	226
Mean	222	216	210	203	192	184	184	182	187
In cold									
	259	266	302	304	158	299	299	283	281
	228	229	272	276	261	258	258	244	234
	233	237	257	279	276	267	267	288	297
	204	221	224	225	271	231	231	227	224
	188	181	183	230	218	219	219	198	190
Mean	222	227	248	263	237	255	255	248	245
In cold + methionine									
	204	240	240	258	262	238	238	230	234
	202	220	207	274	239	244	244	246	220
	192	177	178	178	242	219	219	220	223
	226	254	224	287	258	256	256	231	263
Mean	206	223	212	249	250	239	239	232	235

\* Urines for days 6 and 7 were pooled for analysis

uterus was present, to 10 to 13 days, if in addition daily administration of 1 to 2 mg progesterone was started on the 8th to 11th day of oestrogen treatment. He concluded that the uterus plays a significant role in the formation of relaxin, that relaxation produced by progesterone is mediated by the formation of relaxin in the body and differs from relaxation produced by oestrogens. Pelvic relaxation produced by progesterone has been recorded in the guinea-pig by other workers [for references, see papers by Courrier, and Hisaw *et al*], and the evidence that progesterone plays an active rôle in this species is impressive.

During earlier work on the action of oestrogens and relaxin on the pelvis of the mouse [Hall and Newton, 1947, and other unpublished work], preliminary experiments with progesterone were carried out from time to time, always with negative results. There was even slight evidence that the action of progesterone was inhibitory. It seemed important to establish whether progesterone plays the same rôle in the mouse as it appears to do in the guinea-pig, and the present experiments were planned with this object.

## METHODS

The mice were albinos of the Parkes Strain, they were all virgin females, except in one experiment mentioned in the text. All mice were oöphorectomised at least 2 weeks before treatment was started.

Oestrone (B D H) was dissolved in arachis oil, the volume injected was always 0.05 c.c. Progesterone (British Schering) was used in the form of "Prolution," containing 10 mg progesterone per c.c. of oil. Alternatively, crystalline progesterone was dissolved in arachis oil, the volume injected was always 0.1 c.c. The relaxin extract was prepared from the serum of pregnant rabbits by the method of Abramowitz, Hisaw, Kleinholz, Money, Talmage and Zarrow [1942], as described in a previous paper [Hall, 1948]. The volume of extract injected was 0.2 c.c. unless otherwise stated. All injections were given subcutaneously. The width of the interpubic gap was measured from X-ray photographs [for method see Hall and Newton, 1946], which were made immediately before the start of the experiment (or, in the experiments in which relaxin was given, immediately before the first injection of relaxin) and 24 hours after the last injection. (For a discussion of the time following the injections of relaxin at which the end point should be read, see Hall, 1948.)

## RESULTS

### (1) *Effect of Progesterone on the Symphysis Pubis of Spayed, Oestrogenised Mice*

74 virgin mice, which had never previously received experimental treatment, were used.

(a) 37 mice received daily injections of 1.5  $\mu$ g œstrone plus 1.0 mg progesterone simultaneously for 8 or 9 days. All symphyses remained closed.

(b) 6 mice received 25  $\mu$ g œstrone plus 1.0 mg progesterone daily for 8 days. All symphyses remained closed.

(c) 7 mice received daily injections of 1.5  $\mu$ g œstrone for 19 days and of 1.0 mg progesterone for 17 days, the latter starting on the third day of œstrone treatment. In 6 mice the symphyses remained closed, in 1 mouse an interpubic gap measuring 0.6 mm was recorded.

(d) 9 mice received daily injections of 25  $\mu$ g œstrone and 1.0 mg progesterone simultaneously for 19 days. In 1 mouse an interpubic separation of 1.6 mm was recorded, in 8 mice the separation was 0.0-0.3 mm.

(e) 5 mice received daily injections of 1.5  $\mu$ g œstrone for 24 days plus 1.5 mg progesterone for 19 days, starting on the sixth day of œstrone treatment. All symphyses remained closed.

(f) 10 mice received daily injections of 1.5  $\mu$ g œstrone for 26 days plus 1.5 mg progesterone for 18 days, starting on the ninth day of œstrone treatment. In none of the mice was an interpubic separation greater than 0.5 mm recorded.

In all these experiments, daily administration of 1.0 or 1.5 mg progesterone to oöphorectomised, œstrogenised mice for 8 to 19 days never, with the exception of one mouse in group 4, resulted in interpubic separation of more than 0.6 mm.

Control groups of mice given œstrone alone were not used in these experiments, but in many previous experiments, published and unpublished, oöphorectomised virgin mice have been injected with œstrone for periods up to 22 days. Small interpubic separations are often produced, after two or three weeks' treatment these may reach 0.8 or 1.0 mm. In a group of 11 mice treated with 6.4 to 25  $\mu$ g œstrone daily for 22 days, the average interpubic separation was 0.7 mm [Hall and Newton, 1947]. Separation of 0.5 mm is sometimes recorded after 9 days' treatment.

Therefore, the present experiments provide no evidence that progesterone, in doses up to 1.5 mg per day, either facilitates the action of œstrone on the symphysis pubis of spayed mice, or causes the production of relaxin in the body.

## (2) *Effect of Progesterone on Interpubic Separation produced by Relaxin in Oöphorectomised, Œstrogenised Mice*

These conclusions do not rule out the possibility that progesterone may have a rôle, direct or indirect, in the normal course of pelvic relaxation during pregnancy. Separation of the symphysis pubis can be demonstrated on X-ray photographs from the 13th day of normal

TABLE I.—INHIBITING EFFECT OF PROGESTERONE ON THE RESPONSE OF THE SYNTHESIS PUDIS OF OOPHORECTOMISED MICE TO INJECTIONS OF OESTRONE AND RELAXIN

Group A No progesterone				Group B Progesterone injected				
Serial No of experiment	No of mice used	Treatment	Average width of interpubic separation (mm )		No of mice used	Treatment	Average width of interpubic separation (mm )	
			Immediately before relaxation administration	24 hours after last injection of relaxin			Immediately before relaxation administration	24 hours after last injection of relaxin
7	8	Oestrone 15 µg per day × 9 Relaxin 0.5 cc on day 9 only	0	1.0	8	Oestrone 15 µg per day × 9 Progesterone 10 mg per day × 9 Relaxin 0.5 cc on day 9 only	0	0.2
8	6	Oestrone 25 µg per day × 9 Relaxin 0.5 cc on day 9 only	0	0.9	6	Oestrone 25 µg per day × 9 Progesterone 10 mg per day × 9 Relaxin 0.5 cc on day 9 only	0	0.3

	0	13	0.25	17	0.8-3.6	13						
0		<i>Estro</i> 15 µg per day × 11 <i>Relaxin</i> 0.2 cc per day on days 10, 11										
10	4	<i>Estro</i> 15 µg per day × 8 <i>Relaxin</i> 0.2 cc on days 7, 8	0	2.2	1.9-2.5	5						
11	5	<i>Estro</i> 15 µg per day × 8 <i>Relaxin</i> 0.2 cc on days 7, 8	0	1.9	1.5-2.0	6						
12	6	<i>Estro</i> 15 µg per day × 8 <i>Relaxin</i> 0.2 cc on days 7, 8	0.2	1.7	0.25 (1) other wise 1.8-3.0	6						
13	6	<i>Estro</i> 15 µg per day × 7 <i>Relaxin</i> 0.2 cc per day × 5 (commencing day 3)	0	2.3	1.0-3.2	6						
				4	5	6	7	8	9	10	11	

pregnancy onwards, it proceeds at an average rate of 1 mm per day until parturition on the 19th day [Hall and Newton, 1946] Similar changes can be reproduced in the oöphorectomised animal in the same time interval by injecting daily 0.2 c.c. relaxin extract together with 1.5 µg oestrone after two days' priming with oestrone [Hall and Newton, 1947] A single injection of 0.2 c.c. relaxin given on the last of about eight days' treatment with oestrone alone produces an average interpubic separation of at least 1.0 mm [Hall, 1948] Will the addition of progesterone affect these reactions? Experiments were planned as shown in Table I. Two injections of relaxin on successive days were given, instead of one, in experiments 9-12. This was because individual mice in experiments 7 and 8, Group A, showed only a small reaction to relaxin, and it was thought that more consistent results might be obtained after two days' treatment. The results were similar in all the experiments. In experiment 7, the average width of the interpubic gap produced by a single injection of relaxin following eight days' treatment with oestrone was 1.0 mm (Group A, col. 5), when progesterone was also given during the priming period, the gap measured only 0.2 mm (Group B, col. 10). Increasing the daily dose of oestrone to 25 µg did not alter the results (experiment 8). In experiments 9-12, in which two injections of relaxin were given, the corresponding figures were: Group A, col. 5—1.7, 2.2, 1.9, 1.7 mm; Group B, col. 10—0.4, 0.7, 0.5, 0.3 mm. Furthermore, except for one mouse in Group A, experiment 12, which for some reason failed to react to relaxin, not only were the average figures lower, but the figures for individual mice in the groups which received progesterone were all lower than any figures in the corresponding control groups. Similarly, in experiment 13, in which relaxin was given daily for five days, the average figure for Group A was 2.3 mm and for Group B 0.8 mm.

In all these experiments, therefore, when progesterone was injected daily in addition to oestrone into oöphorectomised mice, the effect of relaxin on symphyseal separation was largely prevented, the result was the same whether the progesterone was given during a preliminary priming period or concurrently with relaxin.

### (3) *Effect of Progesterone in Modifying the Action of Oestrone on the Symphysis Pubis of Oöphorectomised Mice*

Although the roles played respectively by oestrogen and relaxin in the symphyseal reaction are still not entirely clear, it seems possible that relaxin facilitates in some way the action of oestrogen [Hall, 1948]. The effect produced by adding progesterone in the foregoing experiments might be caused by inhibition of action either of relaxin or of oestrone. It is well known that progesterone and oestrogen in certain combinations



are mutually antagonistic, and that progesterone can counteract some of the actions of oestrogen. An attempt was now made to obtain evidence as to whether the failure of the symphysis to open was caused by an inhibitory action of progesterone against oestrone.

The effect on the symphysis pubis of small doses of oestrone is slight, and prolonged treatment is necessary in order to obtain consistent and measurable responses in oophorectomised animals. However, after the pelvis has once reacted to the influence of relaxin, even though the gap may have since closed, it becomes subsequently more sensitive to the action of oestrone, and separation may proceed much faster than is normally the case with oestrogen alone [Hall, 1948]. This acquired sensitivity appears whether the previous separation has been produced by normal pregnancy or by exogenous relaxin. Advantage was taken of it in planning the experiment on 62 mice listed in Table II. The mice in experiments 14, 15 and 17 were oophorectomised when 5 weeks to 2 months old, in a previous experiment they had been treated with combinations of oestrone and relaxin which had produced interpubic gaps of varying width. After the end of the previous experiments all mice had been rested for periods of at least one month, during which time the symphyseal gap had partly or completely reclosed. The average width of the gap at the beginning of the present experiment is shown in the column headed "Day 1". Care was taken that the mice in the two groups of each experiment should be properly balanced with regard to previous treatment, and width of gap at the start of the present experiment. The mice in experiment 16 had all borne at least one litter before they were oophorectomised, in all these mice, the interpubic gap produced by pregnancy had not completely closed.

The effect on the width of the symphyseal gap of 8, 9 and 14 daily injections of oestrone, alone or combined with progesterone, is shown in Table II. In experiments 14 and 15, eight and nine daily injections of 1.5  $\mu$ g oestrone produced an average increase in the width of the gap of 0.7 mm in each case. When 10 mg progesterone was also given daily, the corresponding figure was 0.3 mm. In experiment 16, the injections of 1.5  $\mu$ g oestrone were continued for 14 days, at the end of this period the average increase in the width of the gap was 1.1 mm, in the group which received also progesterone the increase was only 0.5 mm. Raising the daily dose of oestrone to 25  $\mu$ g (experiment 17) did not increase the separation (col 6), while the addition of 1.5 mg progesterone per day inhibited the response to oestrone (col 11). In all these experiments, therefore, progesterone at least partly inhibited the action of oestrone on the symphysis.

TABLE II — INHIBITING EFFECT OF PROGESTERONE ON THE ACTION OF OESTRONE ON SYMPHYSIS PUBIS  
OF OÖPHORECTOMISED MICE  
(The pelvis had been sensitised to oestrone as described in the text)

Serial No of experiment	No of mice used	Treatment	Average width of interpubic gap (mm)			No. of mice used	Treatment	Average width of interpubic gap (mm)		
			Day 1 of experiment	24 hours after last injection	Increase during experiment			Day 1 of experiment	24 hours after last injection	Increase during experiment
14	7	<i>Oestrone</i> 15 µg per day × 8	0	0.7	0.7	7	<i>Oestrone</i> 15 µg per day × 8 <i>Progesterone</i> 10 mg per day × 8	0.2	0.5	0.3
15	9	<i>Oestrone</i> 15 µg per day × 9	0.9	1.6	0.7	9	<i>Oestrone</i> 15 µg per day × 9 <i>Progesterone</i> 10 mg per day × 9	1.1	1.4	0.3
16	7	<i>Oestrone</i> 15 µg per day × 14	1.3	2.4	1.1	7	<i>Oestrone</i> 15 µg per day × 14 <i>Progesterone</i> 10 mg per day × 14	1.5	2.0	0.5
17	8	<i>Oestrone</i> 25 µg per day × 14	0.2	1.3	1.1	8	<i>Oestrone</i> 25 µg per day × 14 <i>Progesterone</i> 15 mg per day × 14	0.1	0.6	0.5
Col 1	2	3	4	5	6	7	8			

## DISCUSSION

The experiments here recorded provide no evidence that progesterone is itself effective in producing symphyseal separation in the mouse, or that it can augment the action of oestrogens on the symphysis or act indirectly by causing the production of relaxin in the body

Hisaw *et al* [1944] found that the maximal response of the symphysis pubis of oöphorectomised guinea-pigs to one injection of progesterone was obtained 72 to 96 hours after the injection, whereas relaxin produced a similar response in 6 hours. The same length of time after one injection of progesterone was needed to bring about the presence of relaxin in the blood of female rabbits. Zarrow [1948] gave progesterone daily to castrated female guinea-pigs from the 8th day of oestradiol treatment, and obtained pelvic relaxation on the 2nd or 3rd day of progesterone treatment. When the two hormones were administered simultaneously from the beginning, relaxation appeared on the 8th day. In oöphorectomised mice a measurable symphyseal response is obtained 24 hours after a single injection of relaxin given on the last of about 8 days' priming with oestrone, the separation increasing with each of 5 or 6 subsequent daily injections of relaxin. Allowing for a time lag between the first administration of progesterone and the formation of endogenous relaxin if such occurred, it would seem that in the present experiments the duration of treatment with progesterone, at least in experiments 3 to 6, should have been long enough to produce a symphyseal response. The daily dose of 1 mg progesterone given in most of these experiments was chosen because that is the dose which will maintain pregnancy in mice oöphorectomised during the latter part of pregnancy [Robson, 1938 *a*, Hall and Newton, 1947]. It is not known how much progesterone is produced throughout pregnancy in the mouse. Robson [1938 *b*] suggested that a concentration of progesterone is present in the pregnant mouse similar to that produced by the daily administration of 1.5 mg of luteal hormone. He did not claim that this quantity is produced daily in the pregnant mouse, but considered that appreciably smaller amounts of the hormone secreted constantly in the body will give the same end result as that produced by daily subcutaneous injection of 1.5 mg.

Qualitative information about the levels of progesterone and of oestrogen present in the pregnant mouse has been provided by Atkinson and Hooker [1945] on the basis of the histological structure of the endometrium of the sterile horn. They found that the level of progesterone was low on the 1st day of pregnancy, rose progressively for 3 days, remained high until the 7th day, then decreased fairly rapidly and reached the initial low level on the 12th day. It remained low for the rest of gestation, but rose steeply on the 1st or 2nd day after parturition. Oestrogen declined during the first 3 days, was almost absent until the

10th day, then rose somewhat and remained at a moderate level for the rest of pregnancy, with a further small increase immediately preceding parturition. Two days after parturition oestrogen activity ceased.

These suggestions are of interest when related to the symphyseal changes which take place during pregnancy. The first sign of separation at the symphysis appears on X-ray films on the 13th day. According to Atkinson and Hooker [1945], the level of progesterone falls to a minimum on the 12th day of pregnancy and, except for a possible slight increase during the last 4 days, remains very low until parturition. The oestrogen level, on the other hand, is highest from the 11th day until parturition. That is, interpubic separation in the pregnant mouse takes place during that period of pregnancy when the level of oestrogen is highest and that of progesterone is lowest.

In a previous paper [Hall, 1948] it was suggested that the action of oestrogen on the symphysis might be fundamental, but that, alone, its effect is small and slow. It is possible that relaxin acts by removing some barrier to, and thus facilitating, the action of oestrogen. Experiments 7-13 (Table I) show that when progesterone was given in addition to oestrogen to oöphorectomised mice the effect of relaxin was largely prevented. Experiments 14-17 (Table II) indicate that this may be caused, not by any direct antagonistic relationship between progesterone and relaxin, but to an inhibitory action of progesterone against oestrogen.

It seems probable, therefore, that the pelvic changes which take place during the second half of pregnancy in the mouse depend upon a proper balance of oestrogen and progesterone in the body. If the ratio of progesterone/oestrogen rises above an optimal level, then the direct action of oestrogen on the symphysis is prevented and the latter hormone is unable to prepare the way for relaxin. There is no evidence that synergism exists between oestrogen and progesterone, such as Courrier finds in the guinea-pig, or that progesterone is the agent which encourages formation of relaxin during the course of normal pregnancy. This implies a species difference in the mechanism of pelvic relaxation in the pregnant mouse and guinea-pig.

### SUMMARY

1 Experiments were carried out on 234 oöphorectomised mice with the aim of discovering whether progesterone plays an active role, such as that described for the guinea-pig, in the mechanism of pelvic relaxation in the mouse.

2 When 1.0 or 1.5 mg progesterone was injected daily for 8-19 days into oestrogenised mice, the interpubic gap as measured on X-ray photographs was 0 to 0.6 mm, i.e. no wider than would be produced by oestrogen alone, and not comparable with the considerable separation which follows relaxin administration or occurs in normal pregnancy.

3 When 10 mg progesterone was injected daily into oestrogenised mice, the effect on symphyseal separation of concurrent or subsequent administration of relaxin was largely prevented

4 When progesterone and oestrone were injected concurrently into mice whose pelvis had been rendered sensitive to oestrogen, the action of oestrone was much impaired

5 The results of the experiments provide no evidence that progesterone itself is effective in producing symphyseal separation in the mouse, or can augment the action of oestrogen on the symphysis, or can act indirectly by causing the production of endogenous relaxin

6 It is suggested that in pubic separation produced by oestrogen and relaxin the action of oestrogen is fundamental, and that the inhibitory effect of progesterone on the separation is caused by antagonism between progesterone and oestrogen

7 The possibility is discussed that the pelvic changes which take place during the latter part of pregnancy in the mouse depend upon the presence of a low progesterone/oestrogen ratio in the body at this time, and that a species difference may exist in this respect between the mouse and the guinea-pig

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PHYSIOLOGICAL AND PATHOLOGICAL RESPONSES IN THE  
BLOOD-VESSELS OF THE LIVER<sup>1</sup> By R. D. SENEVIRATNE  
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EARLY studies on the circulation of the liver yielded a wealth of knowledge yet left many uncertainties. The minute vascular pattern can be seen in its natural physiological setting only in the intact liver of the live animal, and the methods of injection, perfusion and ligation of vessels available to the early investigators were therefore of limited value.

Strong reflected light was used by Malpighi [1686], Gruithuisen [1812] and Muller [1835], and more recently by Ghiron [1912] and Richards and Schmidt [1924], for making observations on intact organs of live animals. Transmitted light from the substage of an ordinary microscope has been used when the position and structure of an organ permitted its arrangement on the stage [Clark, 1909, Zweipach, 1934, McNee, 1931]. Adapted in the Clark-Sandison chamber [Sandison, 1924, 1928, Clark *et al*, 1930], it has a wider field of usefulness, but in neither form is it applicable to the study of the intact liver.

By means of light conducted along transparent rods organs can be transilluminated *in situ*. Basler [1917], Loeffler and Nordman [1925] in studies on the liver circulation, and Florey and Carleton [1926] and Barta [1935] for other tissues, used glass rods. Leiter [1925], Silverman [1925] and Wearn *et al* [1934] used quartz rods.

The purpose of my investigation was to apply this last method, as perfected by Knisely [1936 *a* and *b*, 1937, 1938], (1) to study afresh the intrahepatic circulation, (2) to attempt to clarify some of the existing doubts, particularly with regard to the hepatic artery, and (3) to record the effects of various stimuli on the enormous vascular bed whose response must undoubtedly modify the structure and function of the liver.

#### METHODS

The method illustrated in fig. 1 is a modification of Knisely's technique of transilluminating living structures for microscopic study.

<sup>1</sup> Part of a thesis accepted by the University of London for the Doctorate of Medicine.

Its essence lies in the use of a special mercury vapour lamp and quartz rod with prismatic end, kindly brought to my notice by Dr H C Wright of the Royal Naval Physiology Laboratory, Alverstoke. A straight quartz rod conducts light from the lamp to the liver of the animal arranged on a suitable table, with the microscope focused on it. Ringer's solution passes from a reservoir through a bath maintained at the required temperature to a delivery pipette adjusted to allow the solution to flow over the organ under observation. An injection apparatus clamped to the table is a convenient accessory. The quartz rod, injection needle and pipette for Ringer's solution are held in adjustable clamps. The whole assembly is arranged on a heavy wooden table.

A compact source 250-watt, type ME box lamp [Bourne, 1945] was used throughout. It was cooled by convection [Knusely, 1937] by enclosing it in a stove pipe  $3\frac{1}{2}$  inches in diameter and 24 inches in height. It produces a small concentrated light source of high brightness (18,000 sb) and, unlike the carbon arc, of low heat content, only a small proportion of its energy being radiated in the infra-red region. It has 2 to 3 times the light output of a tungsten filament lamp of the same wattage, reducing further its heating effects. Fluctuations in the voltage of the supply cause smaller variations in its light output than in that of a filament lamp.

The straight quartz rod was 4 mm  $\times$  4 mm in section and 18 cm in length. The receiving end was cut square and the delivery end bevelled at  $45^\circ$ , ground and polished, giving the effect of a prism (fig 2). The rod has no bends and therefore no light is lost. The thin edge occupies very little space, and can be slipped under the liver with very little disturbance and small operative trauma.

Heat may reach or leave the transilluminated object by conduction, convection and radiation. Conduction is eliminated by the use of a sufficient length of quartz which has a very low thermal conductivity. The heat emitted by the type of lamp used in my experiments is small, and, with the cooling arrangement used, heat does not reach the object by convection or radiation in sufficient quantity to heat it. The illuminated structure, however, absorbs some of the light applied to it and transforms it to heat. It is very likely that this heat is dissipated by the copious blood-flow of the liver as rapidly as it is formed, but to obviate any possible heating effect, a continuous flowing solution, isotonic and isothermal with the tissues, was applied to the liver.

The efficiency of temperature control was tested by taking the temperature of the delivery end of the quartz rod and transilluminated liver with a delicate thermocouple before and after 1 hour of continuous transillumination. No change of temperature was found.

A wooden table 8 inches  $\times$  20 inches and 4 inches high was found most convenient as an operation table. A shallow white enamelled



tray  $5\frac{1}{2}$  inches  $\times$  12 inches with sides  $\frac{1}{2}$  inch high was placed on the table, and the animal was supported in this tray on a cork mat which raised it clear of waste solutions. A cotton wick at one corner of the tray siphoned away any accumulation of fluid.

The transilluminated liver was observed with a Winkel-Zeiss microscope from which the mechanical stage had been removed. Most of the observations were made under 10 and 40 objectives with  $\times 5$  and  $\times 10$  eye-pieces. A Zeiss water-immersion lens was used when very high magnification was desired.

*Animals* —In all 98 frogs (*Rana pipiens*), 48 mice and 112 rats were studied.

*Surgical Procedure in Animals* —The frog was pithed, or anæsthetised with Nembutal injected into the dorsal lymph sac, and pinned on the cork mat. The abdomen was opened by a left paramedian incision extending from half-way down the abdomen to the level of the xiphisternum, whence a transverse incision to the left allowed a flap of abdominal wall to be turned down, giving excellent exposure of the liver. When a wider exposure was required, the anterior abdominal vein was tied at the symphysis pubis and a V-shaped incision was made with its point at the symphysis and its limbs at the shoulders. The V flap was lifted up with the anterior abdominal vein, through which injections could be made directly into the portal vein.

All mice and rats were anæsthetised with Nembutal intraperitoneally and the anæsthesia maintained during all procedures. The midline incision extended from the xiphisternum to about the mid-point of the abdomen, and from its centre a transverse cut to the right or, when exposure of the spleen was required for injection, to the left allowed a triangular flap of abdominal wall to be turned up over the costal margin and, if necessary, fixed by a stitch.

The liver having been exposed, the animal was laid on the cork mat of the operation table. The delivery tip of the illuminating rod was gently slipped under the edge of the liver and the lamp, switched on fifteen minutes previously to ensure maximum illumination, arranged with its window opposite the receiving end. A gentle drip of Ringer's solution at the correct temperature was commenced. Finally, the microscope was placed with its objective over the liver. The intensity of illumination could be varied by moving the lamp closer to or away from the rod. By moving the rod, the microscope, or both, different parts of the liver could be observed.

## RESULTS

### 1 *Normal Blood-flow in the Liver*

An arresting picture of vivid colours and rapid movement is seen. The liver parenchyma is a transparent red in the thicker parts and

## DESCRIPTION OF FIGURES

FIG 1 —The apparatus for transilluminating the liver of living animals

- |  |  |
|--|--|
| 1 Lamp   | 6 Delivery pipette for Ringer's solution |
| 2 Stove pipe enclosing lamp                                  | 7 Enamel tray                            |
| 3 Quartz rod   | 8 Operating table                        |
| 4 Ringer's solution  | 9 Microscope                             |
| 5 Water bath for regulating temperature of Ringer's solution | 10 Injection apparatus                   |
|  | 11 Adjustable clamps                     |

FIG 2 —Quartz rod, showing prismatic delivery

FIG 3 —Portal vein entering the portal tissue accompanied by the hepatic artery

FIG 4 —A highly magnified drawing to show the appearance of the sinusoids and their relation to the liver cells as observed directly in transilluminated livers

FIG 11 —A diagrammatic representation of the sequences of events seen after intraportal injection of Evans Blue

- Immediately after injection—dye entering portal branches
- 2 minutes after—dye has spread uniformly through all the sinusoids and vessels
- 5 minutes after—blue stain appearing outside the central hepatic veins
- $\frac{1}{2}$  hour after—tissue and cells round central hepatic vein deeply stained

FIG 13 —The appearance of a microscopic field in the transilluminated liver of a live rat before administration of carbon tetrachloride

FIG 14 —The appearance under the microscope of a transilluminated rat liver 6 to 8 hours after carbon tetrachloride. The cells along the central hepatic vein are showing degenerative changes

FIG 15 —The appearance under the microscope of a transilluminated rat liver 24 hours after carbon tetrachloride. The area of degeneration round the central hepatic vein is wider and the changes more marked

FIG 16 —A semi diagrammatic reconstruction of the distribution and connections of blood vessels in the hepatic lobule, from direct observations of the transilluminated livers of live animals

Hepatic artery—black.  
Portal vein—light

A copious capillary plexus round the portal vein has been omitted for clarity

- Large hepatic arterial branch
- Large portal venous branch
- Small hepatic arterial branch
- Small portal venous branch in portal tract
- Connection between hepatic artery and portal vein in portal tract
- and 7 Arterioles opening into the sinusoids in the periphery and central zones respectively of the liver lobule
- Sinusoid
- Hepatic venous radicle in centre of lobule
- Hepatic cells
- Specialised liver cells along the central hepatic vein

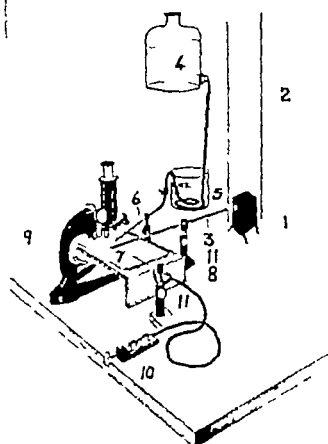


FIG 1



FIG 2

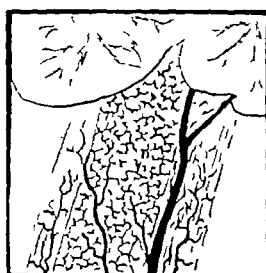


FIG 3

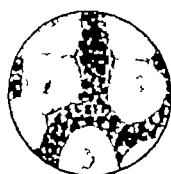


FIG 4



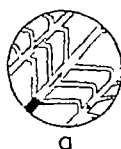
FIG 13



FIG 14



FIG 15



a



b



c



d

FIG 11

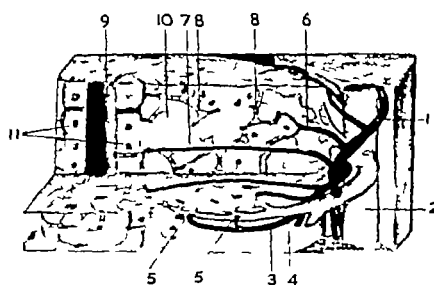


FIG 16



pale yellow at the edges Individual cell margins are clearly defined The blood-vessels and sinusoids appear in shades of red, varying from orange in the minute arterioles and venules to deep crimson in the larger ones The various vascular components can be distinguished by the direction of flow, from the larger to the smaller branches and sinusoids in the portal vessels and in the opposite direction in the hepatic veins, also by the size, type of branching and rate of blood-flow

The presence of copious black pigment granules in the liver of the frog, particularly during the breeding season, obscured the vascular pattern, but by selecting small pale frogs much of this difficulty was avoided After some experience one was able to follow the blood-flow in the moving liver of the rat and mouse without difficulty and respiratory movements ceased to be a hindrance Methods such as cutting the phrenic nerve or holding the liver in a wire snare must cause gross departure from the physiological and so were not employed

*The Frog*—Histological preparations convey little of the dynamic and rapidly moving picture seen in the living organ, or the delicate and prompt vascular responses of which it is capable There is no clear-cut lobular pattern

The portal vein enters the liver with the hepatic artery and bile duct, wrapped in a sheath of loose connective tissue Narrow arterial branches wind round it and break up into a rich capillary plexus in its walls (fig 3) The portal blood has a slight purplish tint compared with the bright red in the arteries The rapid rush of blood in the arterial branches and turning and twisting capillaries contrasts sharply with the comparatively slow, smooth flow in the broad portal stream In the liver, portal branches, accompanied by slender arteries, pass towards the periphery of the organ within a bed of connective tissue which separates them from the liver parenchyma No sinusoids come off the main branches and very few from the smaller ones Near the termination, short stout branches arise and immediately break up into bunches of sinusoids Less commonly, single sinusoids are given off The termination of the portal branches is rather abrupt Instead of narrowing gradually they break up into two or three divisions, which immediately end in radiating sinusoids Portal branches seldom reach the extreme periphery of the liver but end some distance from the liver edge Blood-flow in the active areas is smooth and continuous, but in the less active areas, or when the circulation is slowing down, it becomes jerky or intermittent, moving forward sharply with each systole and slowing down during diastole Just before complete arrest of flow a slow oscillation is often seen

The hepatic venous radicles commence at the extreme edge of the liver and converge to form larger branches Some of these branches course just within and parallel to the liver edge Unlike the portal vessels the hepatic veins gradually widen as they receive more branches

Sinusoids generally enter singly at regular intervals even into the larger veins. There is hardly any connective tissue separating the vessel wall from the liver cells. A mantle of cuboidal, solid-looking liver cells, unlike the polygonal or spherical cells seen in irregular anastomosing columns in the rest of the lobule, encircles the vessel. Blood-flow is normally smooth and continuous, but may become intermittent or oscillate like the portal flow under similar conditions.

The sinusoids form a complex network between the portal and hepatic veins. The number of visible sinusoids and their calibres vary not only in different areas but also in the same area at different times. Each sinusoid may be anything from a barely visible cleft to a channel 2 to 3 red cell diameters wide. A definite membrane appears to line the sinusoids. It cannot be seen in its whole length, but the red cells in stagnant sinusoids give the clear impression of being packed in a transparent tube (fig. 4). In places this lining membrane is lifted off the liver cell columns, suggesting that the sinusoidal wall is loosely applied to the surrounding liver parenchyma, but there is no other suggestion of a perisinusoidal space. An irregular intermittency in activity characterises different areas. A very large percentage of sinusoids may be inactive for long periods of time. In the quiescent areas the sinusoids are either collapsed and empty, or distended with blood. In the active areas blood cells flow in closely packed columns in the dilated sinusoids, but in the contracted ones individual red cells appear to squeeze through in single file. The rate of flow does not, however, appear to depend entirely on the calibre of the sinusoids. Through contracted ones it is often very rapid, suggesting an increased inflow or a reduction of resistance at the outflow. Normally blood leaving the portal veins by the sinusoids reaches the hepatic vein, but when the circulation is slowing down, the flow in a few sinusoids bordering the portal branches is reversed so that they empty back into the portal vein. I could see no indication of valves or localised contractions, but the sinusoid appeared to narrow along its whole length like a thin-walled collapsible tube.

The hepatic artery on entering the liver divides into branches which accompany the portal veins. Being very slender and the blood-flow extremely rapid they are not easy to see, but on careful observation, particularly when the circulation is slowing down, they can be seen accompanying the portal radicles in straight channels, gentle curves or wide spirals. The blood-flow is so rapid that in the smallest branches red blood cells flash across the field in single file, and the actual vessel wall is distinguished only when the circulation slackens.

Many connections exist between the arterial and venous blood. Often short branches pass from the hepatic artery to the accompanying portal vein, or enter the lobule to flow into a sinusoid usually at the periphery but not uncommonly at the centre of the lobule. Sometimes

an arteriole stretches across the lobule to open into a portal vein on the opposite side, or less frequently into an hepatic vein. Tiny arterial branches may pass through the liver substance and join with sub-capsular arteries. I could detect no territories exclusively supplied by arterial or venous blood. Everywhere the sinusoids appeared to receive mixed arterial and portal blood.

*Mouse and Rat*—The liver of the rat and mouse shows a more definite lobular pattern. The portal radicles mark out roughly polygonal areas of liver cells round the central hepatic vein. The general outline and the type of branching of the individual vessels and sinusoids are essentially the same as in the frog, but the terminal branches and sinusoids are finer and the whole picture more delicate and clearly

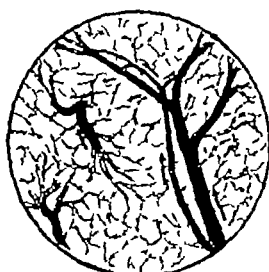


FIG 5

FIG 5—An arteriole running with a portal venous branch and connecting with it by arteriovenous anastomoses



FIG 6

FIG 6—An arteriole running across a hepatic lobule to open into the sinusoidal bed near the central hepatic vein

defined. Blood-flow is generally more rapid, and the number of active vessels and sinusoids in any one field is far greater than in the frog's liver. The slender hepatic arterial branches are seldom seen owing to the extreme rapidity of blood-flow and small size of the red cells, but at the end of an experiment, when the circulation is slowing down or when it is deliberately retarded, they come into view. They course with the portal radicles in gentle curves or wind round them in wide open spirals. Many types of anastomoses between the arterial and venous blood are seen. The commonest is the direct connection by short branches between the hepatic artery and the accompanying portal vein. Fig 5 is a drawing of one of these. Near the termination of the portal vein its accompanying arteriole gave off a short branch which curved back to open into the vein. The rush of arterial blood stirred up the sluggish portal stream into a turbulent whirl at its point of entry. Beyond this the portal flow was quite rapid, as if the momentum of the arterial blood had given it an impetus. The continuation of the arteriole appeared to connect again with the portal vein a little farther on. Another type of anastomosis is depicted in fig 6. Connections were also seen between

the arterioles and sinusoids in the periphery of the lobule. Sometimes arterioles appeared to lose themselves amongst the sinusoids in the immediate vicinity of the central vein.

## 2 *Effect of Obstructing the Portal Vein, Hepatic Artery and Hepatic Vein*

Ligaturing the portal vein of the frog leads to little or no disturbance of flow in the portal or hepatic vessels or in the sinusoids. In the rat a temporary slowing of the blood-flow and slight narrowing of the portal and hepatic veins occur in irregular segments so that their outlines



FIG 7



FIG 8



FIG 9



FIG 10

FIG 7—Appearance of a microscopic field which includes a portal venous branch and hepatic venous radicle immediately after tying the portal vein—slight narrowing and irregularity of the vessels

FIG 8—Same field as fig 7 after the hepatic artery too, has been tied—irregularity and fragmentation of the portal and hepatic veins and complete collapse of the sinusoids, except a few in the mid zonal region

FIG 9—Appearance of a transilluminated rat liver before tying the hepatic vein.

FIG 10—Same field as fig 9, five minutes after tying the hepatic vein.

become uneven (fig 7). But within two or three minutes the circulation returns to normal and continues without further alteration. The arteriovenous anastomoses appear to be sufficient to maintain a normal blood-flow.

When the hepatic artery alone is tied a similar sequence of events occurs.

With simultaneous ligature of the portal vein and hepatic artery there is an immediate and striking reduction of blood-flow. In the frog a slow trickle of blood continues for several minutes, sometimes for an hour. But in the rat the portal vein empties and collapses in irregular segments, it is narrowed to a mere line in some parts, and dilates into irregularly fusiform pools of stagnant blood in others. The sinusoids are completely emptied at first, but a slow back-flow from the hepatic veins fills a few in the mid-zonal regions. The hepatic veins too, after a period of oscillation and sometimes reversal of flow, empty completely except for little bead-like pools of stagnant blood at irregular intervals along their course. This sequence of events generally takes three to five minutes, after which there is complete stasis. The whole liver in the live animal is pale and bloodless except for little collections of



stagnant blood along the portal and hepatic veins and in a few mid-zonal sinusoids (fig 8)

To observe the effects of obstructing the outflow from the hepatic vein, the inferior vena cava was tied just below the diaphragm in the rat, and the median vena cava between the liver and the sinus venosus in the frog. The picture was the same in both cases. The flow in the hepatic veins slowed immediately and, after a period of oscillation for two or three minutes, stopped completely. But in the portal venous radicles, hepatic arterial branches and sinusoids, the flow continued for about five minutes, during which the sinusoids became stuffed with red cells. Finally blood-flow ceased with all vessels equally dilated and full of blood, the sinusoids were increased to two or three times their normal width (figs 9 and 10), and many which were previously collapsed and invisible now came into view packed with red cells. A few hæmorrhages developed, most often in the centrilobular regions. The final picture showed an enormous dilation of vessels and sinusoids seen under no other circumstances.

*Summary*—Obstruction of the portal vein or hepatic artery alone causes only a temporary reduction of blood-flow, for anastamotic connections between the two soon re-establish the circulation.

When both vessels are obstructed simultaneously in the rat the blood-flow is arrested and the liver drained of blood, but in the frog a slow circulation through collaterals continues for a considerable time.

Obstruction of the hepatic veins in both rats and frogs causes marked dilation of all vessels and complete arrest of circulation.

### 3 *Effect of Eck Fistula on Hepatic Circulation*

The operations for establishing Eck fistulæ in Albino rats were kindly performed by Dr K. K. Cheng, of the Graham Research Laboratories, UCH Medical School, according to the method of Whitaker [1946]. No abnormality of any kind could be detected in any of the animals in the liver circulation, which appeared to be completely maintained by the hepatic artery.

### 4 *Effect of Portal Embolism*

A suspension of starch in 5 per cent gum acacia was injected into the portal circulation of frogs and rats. The individual starch granules varied from about 5–40  $\mu$  in diameter.

When a small quantity (0.25 c.c. in the frog or 0.55 c.c. in the rat) was introduced slowly no change in the circulation could be detected, although starch granules were seen to be arrested at various points, the larger ones in the terminal branches of the portal vein and smaller ones in the sinusoids. Very few large granules reached the central

hepatic vein Occasionally a short segment of sinusoid behind an obstruction was inactive and dilated, but blood appeared to get round such small obstructions easily by alternative channels

When larger quantities were injected, starch granules accumulated behind the first obstruction till considerable lengths of portal vein with its branches and sinusoids were packed with them The circulation for some distance around the obstructed area came to a standstill, but remained quite active beyond it and in the hepatic vein draining the area

### 5 *Effect of Stimulating Autonomic Nerves*

Rats were employed in this experiment

*Sympathetic*—Since the sympathetic nerves pass to the liver along the blood-vessels in the portal fissure, the whole portal vascular bundle was carefully dissected out and placed across shielded electrodes and stimulated by a tetanising current The effect on the circulation increased up to a maximum limit, with increase of the strength and duration of the tetanus

On stimulating the sympathetic plexus the whole field under observation blanches, due to contraction of the sinusoids, some of which completely disappear from view There is no perceptible effect on the portal or hepatic veins On a few occasions when the hepatic arterioles were under observation they appeared to contract Blood flow, however, continued very actively through the narrow sinusoids and other vessels, suggesting either a systemic rise of blood-pressure or a reduced resistance at the outflow When the stimulation was discontinued the vessels returned to normal in about 10 or 15 seconds If the stimulation was repeated the same series of changes occurred The vasoconstrictor effect was considerably reduced after subcutaneous injection of 2 mg of ergotamine tartrate

*Parasympathetic*—The parasympathetic nerve-supply to the abdominal viscera is through the vagi Near the cardia of the stomach these nerves break up into several branches, which can be separated from the oesophagus immediately below the diaphragm, ligatured, and laid across the shielded electrodes distal to the ligature

Tetanising currents of increasing strength and duration up to 60 seconds fail to produce any visible change in the pattern or rate of flow of the hepatic circulation

### 6 *Localisation of Dyes within the Liver*

The results in rats and frogs were very similar

*Evans Blue*—On injecting a 2 per cent Evans Blue solution directly into the portal circulation it appears immediately in the portal radicles, marking out the liver lobules vividly, then spreads slowly through the

sinusoids into the central hepatic veins. For a minute or two the portal veins and peripheral zones of the lobule are darker than the central region, but soon the colour begins to get lighter in this area as the dye mixes with the circulating blood and reaches the same intensity all over. Soon afterwards a striking phenomenon is observed. In three or four minutes a blue coloration appears just outside the hepatic veins in what seems to be a thin layer of connective tissue, and also in the hepatic cells lying immediately alongside. The colour here gradually increases in intensity, while the portal veins and the rest of the liver sinusoids now fade to a uniform pale blue. In half an hour this localisation is still very striking. Kupffer cells at intervals along the sinusoids seem to be absorbing the dye too. Fig 11 represents diagrammatically the sequence of events.

When the dye is injected into the general circulation, a uniform blue tinge spreads all through the liver lobules, commencing in the portal tracts. However, in 5 or 6 minutes the distribution of the dye described above appears, and a picture very similar to that produced by intra-portal injection is seen.

Dye was also injected into groups of rats that had received 0.25 c.c. of carbon tetrachloride subcutaneously respectively 6 hours and 24 hours previously, and showed centrilobular damage in the liver. The spread and localisation of the dye followed as before, but a wider zone, including the whole damaged area, took a deeper stain. The maximum concentration was still in the layer of cells immediately bordering the central hepatic vein.

When *India ink* in 20 per cent suspension was injected intraportally, it appeared first in the portal areas and periphery of the lobules, but rapidly spread through the sinusoids to the central hepatic veins. In about 60 seconds the whole field was so black that individual vessels and sinusoids could scarcely be seen. In a very short time, however, the central zones began to clear. The outlines of the sinusoids and vessels reappeared and particles of ink could be seen mixed with blood-cells. At irregular intervals little collections of dye seemed to be taken up by the Kupffer cells in the lobule. The sinusoids were uniformly dilated. In about 10 minutes the hepatic veins and the sinusoids in the central zones were clear of dye except for a few particles which circulated in the flowing blood, but the peripheral sinusoids and portal areas retained a large proportion of the ink within the vessels. At no stage did ink appear outside the blood-vessels. This picture persisted for one hour or more during which the liver was under observation.

*Trypan Blue* in 6 per cent solution produced a picture very similar to that with Evans Blue, but the intensity of staining of the central zones of the liver lobules was less, and in about half an hour the dye seemed to have diffused uniformly over the whole liver lobule.

*Summary*—Soluble dyes circulating in the liver appear to leave the sinusoids in the central zones in greatest concentration, and accumulate in the liver cells along the central hepatic veins. There appears to be either a higher permeability of the sinusoids in this area, or an inherent capacity of its cells to remove and concentrate such dyes. Particulate matter, such as India ink, does not pass out of the vessels.

### 7 *Effect of Hypertonic Solutions*

When a 30 per cent solution of *sodium chloride* was slowly injected into the tail vein of the rat, no appreciable effect on the liver circulation was seen until 3 c c or more were given. With larger quantities up to 5 c c the sinusoids dilated slightly for about 10 or 15 minutes, after which they returned to normal. If the injection were given directly into the portal vein, with the first drop or two there was an immediate contraction of the sinusoids—most evident in the peripheral zones of the lobule—which lasted till the whole amount had been introduced. Then the vessels began to recover rapidly, and in about 5 minutes a slight dilatation was noticed in all areas. This too disappeared in about 15 minutes and the liver circulation returned to normal.

A 50 per cent *urea* solution produced very similar, though more severe, changes. The constriction of the sinusoids with intraportal injection was almost violent. The dilatation following both intraportal and intravenous injection was very striking and lasted throughout the period of observation, generally about 3 hours.

*Glucose* in 50 per cent solution produced effects almost identical with those after 30 per cent saline.

The initial constriction which results from intraportal injection appears to be the response of the sinusoids to the direct action of an irritant in high concentration, and the succeeding dilatation part of a general vascular dilatation which is an attempt to accommodate the increased volume of blood consequent upon the osmotic action of the irritant. The return towards normal in a short time is perhaps due to dispersal of this increased volume in freshly opened capillaries throughout the body, so that the effect on the liver is too slight to be appreciated.

### 8 *Effect of Respiratory Disturbance on the Liver Circulation*

Under Nembutal anaesthesia a glass cannula was inserted and tied into a rat's trachea. A screw clip on a rubber tube attached to the cannula served to regulate the size of the only airway now available. The liver was then exposed and arranged for transillumination.

When the airway was reduced and respiration became deep and laboured, the liver paled all over. The maximum effect appeared in the sinusoids. Slight narrowing of the portal and hepatic veins was

also seen, although it was not a marked feature. When the respiratory effort was very strong, the narrowing of the sinusoids increased during inspiration and decreased during expiration, allowing some blood to re-enter. On opening the airway so that the respiration was no longer embarrassed, the hepatic vessels and sinusoids rapidly returned to normal. When the experiment was repeated several times at short intervals, the liver circulation went through the same changes each time.

To test the effect of breathing pure nitrogen, or air with low oxygen tension, the apparatus shown in fig 12 was set up. The tracheal cannula was connected through a soda-lime container (A), for absorbing carbon dioxide in the expired air, with the bottle containing pyrogallol

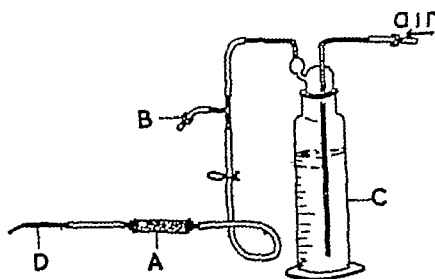


FIG 12 —Apparatus for investigating the effects of respiration and anoxia on the liver circulation.

- A Soda-lime container
- B Screw clip on side arm of T tube
- C Bottle containing pyrogallol.
- D Cannula for insertion into trachea

(15 per cent in 50 per cent sodium hydroxide). The connecting rubber tube was fitted with a side-arm controlled by a screw clip (B). When B was closed, the animal breathed air drawn through the pyrogallol and therefore received only nitrogen. By opening B oxygen-containing air could be admitted.

When the rat was made to breathe pure nitrogen, respiration became somewhat deeper. Slight blanching of the whole liver, increasing at every inspiratory effort, was at first seen, but in about 2 minutes respiration ceased, the sinusoids dilated all over and filled with blood and the animal died with widely dilated sinusoids. When an animal was allowed to breathe air with a low oxygen concentration, respiration again became deep and the sinusoids contracted and emptied with each deep inspiration. In about 10 minutes, however, they began to dilate, although the central zone sinusoids still narrowed during inspiration. The dilatation continued to increase till in half an hour all the sinusoids were widely distended. Deep inspiration still seemed to influence the calibre of the sinusoids. But after one hour the dilatation seemed to be permanent. Neither the phases of the respiratory cycle nor the

depth of respiration caused any change. Blood-flow in the sinusoids was very slow, and in some there was complete stasis. Even when pure air was readmitted to the lungs the vessels remained dilated and stagnant. Permanent or at least lasting damage appeared to have resulted from one hour of breathing air with low oxygen tension. But if the animal was allowed to breathe pure air after 10 to 15 minutes of anoxia, the liver circulation rapidly returned to normal.

*Summary*—Not only does deep respiration narrow all the liver vessels, but each inspiratory effort further constricts them, while expiration produces some relaxation and re-entry of blood. Low oxygen tension in the inspired air results in a dilatation of sinusoids, which at first react to the phases of respiration, but after about an hour appear to be permanently damaged and remain dilated.

### 9 *Effect of Hormones and Drugs on the Liver Circulation*

(1) *Adrenalin*—Two drops of a 0.1 per cent adrenalin hydrochloride solution dropped on the surface of the liver causes the sinusoids to contract almost immediately. Some become so narrow that blood-cells cannot enter, but in the majority blood-flow remains very active and the rate of flow seems to be faster than before. These observations suggest an increased pressure at the inflow or a diminished resistance to the outflow. There is no noticeable effect on the portal or hepatic veins. Hepatic arteries could not be seen because of constriction. Frogs, mice and rats reacted in the same way. The effect lasted for about 15 to 20 minutes, after which the circulation returned to normal.

Injection of 0.2 cc of a 0.1 per cent adrenalin hydrochloride solution into the portal vein (through the anterior abdominal vein of the frog or a mesenteric vein of the rat) produces very similar changes though of shorter duration, usually about 10 minutes.

Subcutaneous injection gave the same effects, but these were much less marked, suggesting that the action of adrenalin on the liver vessels is mainly direct.

(2) *Acetylcholine*—Acetylcholine in 0.1 per cent solution when dropped on the liver surface of frogs and rats produces no immediate visible effect on the liver vessels. Intraportal injections, however, induce a slight constriction of sinusoids for about 2 minutes. With all these methods there followed in 3 or 4 minutes a slight slowing of blood-flow and a general appearance of reduced activity. The heart-rate was slowed. A subcutaneous injection of 0.1 cc to frogs and 0.2 cc to rats of eserine salicylate in a 0.1 per cent solution was given 5 minutes before acetylcholine to inactivate choline esterase. With local application or intraportal injection there was now a constriction of sinusoids lasting 2 or 3 minutes, followed by a gradual dilatation. In about 10 minutes this dilatation was well marked. The rate of blood flow

was now very slow. Many of the sinusoids were stagnant and full of blood. With subcutaneous injection the initial contraction was not seen, but the subsequent events were the same. During this time the heart became very slow and feeble. After about 30 minutes the heart began to improve in rate and force and the liver circulation began to get more active. The sinusoids emptied and returned towards normal. Except the initial contraction, the whole sequence of events seemed to run parallel with the rate and the force of the heart, and the circulatory changes in the liver appeared to be, in part at least, a reflection of the feeble general circulation. The initial contraction was possibly the result of a direct action of the drug on the liver vessels.

(iii) *Histamine* —Direct application to the liver surface or intraportal injection of histamine produced within a minute or two marked dilatation of the sinusoids. No change was detected in the other vessels of the liver. Although the sinusoids were dilated and full of blood the rate of flow was retarded, suggesting increased resistance to the out-flow, or an inflow with reduced pressure due to the vasodilator effects of histamine on the rest of the circulation. The effect lasted generally half an hour or longer.

(iv) *Pituitrin* —No appreciable change in the intrahepatic circulation could be seen with pituitrin given locally, intraportally or subcutaneously.

### 10 *Effect of Trauma on Intrahepatic Blood-flow*

With the liver exposed and arranged for observation, the left thigh was crushed with a crushing clamp in two places till the bone was fractured. There was an immediate paling of the whole field with marked narrowing of the sinusoids. The portal and hepatic veins, too, appeared to narrow in some of the animals. Blood-flow, however, continued unaltered in rate, though the quantity of blood passing through the liver must necessarily have been reduced. The effect on the hepatic arterioles could not be gauged as, even normally, they are only about one red cell diameter wide and the flow rapid. For 10 to 15 minutes the vessels continued in this state and then began to dilate gradually. In half an hour the sinusoids were widely dilated, and the portal and hepatic veins had at least returned to normal. The flow now began to slow down. This dilatation and slowing progressively increased, till in about one hour the sinusoids were packed with red blood cells and in places little varicosities full of stagnant blood were seen. Occasionally red cells were found outside the vessels forming little pools. Many of the sinusoids were now quite inactive, while a slow blood-flow continued in a few. Some of the rats died in about one and a half hours, but others lived for over three hours with this picture unaltered.

### 11 *Effect of Heating or Cooling the Body Surface*

Heat and cold were applied during transillumination to various parts of the body by means of test-tubes containing water at the required temperature, also with cotton swabs dipped in hot or cold water

Application of the test-tube or swab at 20° C either to the right hypochondrium, chest or thighs did not produce any visible change in the liver circulation. When 30° C was applied to the chest or right hypochondrium there was no immediate response, but if the heating was continued a slight dilatation of the sinusoids resulted. From the thigh this temperature produced no result, suggesting that the response was due to heat conducted to the liver through the body wall or to a segmental reflex. 40° C applied to the right side of the chest caused an immediate contraction of sinusoids all over the liver. A slight but definite contraction was seen from the thigh too. But in neither case was this contraction maintained even though the heating was continued. Within 60 seconds the sinusoids began to dilate and in two or three minutes were very wide. On removing the heat the dilatation continued for 10 to 15 minutes, then slowly returned to normal. Temperatures of 50° C, 60° C and 70° C resulted in the same sequence of events.

Cold was applied in a similar manner. 10° C applied to the chest or thigh caused a contraction of all the sinusoids, which remained as long as the cooling was continued. On discontinuing the cooling the sinusoids rapidly returned to normal. Similar results were obtained with 4° C.

### 12 *Effect of Mechanical Stimuli applied to the Liver*

When the surface of the liver was gently stroked with a blunt ended glass rod, a wave of constriction of the sinusoids followed in the wake of the rod, to be replaced immediately by dilatation. No change was noticed in the portal and hepatic venous radicles. With repeated strokes the dilatation gradually increased. The sinusoids were packed with red cells and the flow was slower. At this stage some dilatation of the portal and hepatic veins was appearing. If the stroking was continued or pressure with the rod increased, red blood cells appeared outside the sinusoids at one or two spots and spread out over a considerable area. The blood-flow was further retarded, ceasing altogether in a large portion of the sinusoidal bed. Any further trauma only increased the blood outside the vessels till the whole picture was completely obscured by hæmorrhage.

Light pressure with a glass slide on the surface of the liver caused a general blanching, with collapse and narrowing of many sinusoids. On removing the pressure the sinusoids soon filled with blood and began to



dilate, till in 2 or 3 minutes they were quite wide. The blood-flow slowed down considerably. Repeated application of pressure resulted in changes very similar to those produced by stroking.

### 13 *Effects of Heat and Cold on the Liver*

Saline at 20° C dropped on the surface of the liver produced no change in the hepatic circulation. With saline at 10° C the sinusoids began to contract immediately, and in about 60 seconds were markedly contracted. They remained so for 4 or 5 minutes, then returned to normal. Saline at 4° C gave rise to a similar response, but the swing back appeared to overshoot the normal and lead to slight dilatation lasting 10 to 15 minutes. Repeated application of cold resulted in a repetition of the above changes each time, but after 5 or 6 applications the sinusoids remained dilated although still responding by contraction to cold.

Warm saline at 30° C applied to the liver produced a dilatation of sinusoids which lasted about 10 minutes. When saline at 40° C was dropped on the liver the sinusoids contracted promptly, but within 60 seconds began to dilate. At 50° C, 60° C and 70° C the immediate contraction was very marked, but a lasting dilatation soon followed. This degree of heat appeared to have caused permanent damage to the sinusoids, resulting in dilatation. The flow through the dilated sinusoids was at first rapid but soon became very slow.

### 14 *Effect of Hæmorrhage*

Bleeding from the jugular vein at the rate of about 1 c c a minute produced no obvious effect till about 3 c c of blood had escaped. Then the sinusoids began to contract, and as the bleeding proceeded the portal and hepatic branches contracted too. By the time 4 c c of blood was lost the liver was blanched, many sinusoids had contracted and disappeared from view, a few narrow ones maintained a slow trickle of blood. The portal and hepatic veins were now very narrow and their blood-flow retarded. Thin hepatic arterioles could now be seen, in which the blood was still very actively flowing, although much slower than under normal conditions. These changes progressed while the bleeding was continued till about 5 c c had been lost. Most rats were dying at this stage. The portal and hepatic vessels were very narrow, their calibres uneven, and blood-flow extremely slow. Very few sinusoids were visible, and these appeared as interrupted lines containing a few red cells.

### 15 *Effect of Toxic Agents*

A *Carbon Tetrachloride* —When a cotton swab soaked in carbon tetrachloride was held to the nose of mice and rats a slight contraction

of the sinusoids appeared immediately, but in two or three minutes they had returned to normal. If the inhalation was continued for about 15 minutes or longer they began to dilate.

Rats averaging 300 g in weight were given 0.25 c.c. of carbon tetrachloride subcutaneously. In about 15 minutes the sinusoids began to dilate. The animals were observed for 3 hours, by which time the dilatation was definite though not very great. Blood-flow continued actively. The portal venules, hepatic arterioles and hepatic venous radicles showed no appreciable change. The liver parenchyma appeared normal (fig 13). Other rats were examined 6, 8 and 24 hours respectively after carbon tetrachloride. At 6 to 8 hours the sinusoids were still dilated and the blood-flow active, but changes in the central zone of the lobule were beginning to appear. Here and there cells appeared greyish, granular and rather opaque, their outlines were blurred and contrasted markedly with the translucent yellow cells of the peripheral zones (fig 14). In 24 hours the cell damage was severe, cell margins had disappeared and the damaged area had increased in width. The whole area appeared a mass of structureless grey debris (fig 15). The sinusoids passing through it were now compressed, most of them completely obliterated. Through a few irregular sinusoids a trickle of blood was reaching the central hepatic vein. In the peripheral zones the cells showed no visible change, the sinusoids remained dilated and blood-flow continued actively. The portal and hepatic veins and hepatic arteries appeared normal.

When 0.025 c.c. of carbon tetrachloride was injected directly into the portal vein mottled white areas appeared in the liver immediately. Microscopically, large scattered areas, mainly periportal, became almost bloodless through extreme contraction of sinusoids, small portal radicles and their terminal branches. The central vein with the sinusoids immediately around stood out in sharp contrast. After a few minutes the sinusoids began to dilate, and in about 15 minutes were widely dilated. This high concentration of carbon tetrachloride is grossly unnatural, and the initial contraction appears to be a non-specific response to a strong irritant. A similar reaction is seen with other irritants.

**B Chloroform**—Chloroform gave similar results in rats to carbon tetrachloride, though less marked. It appeared to be more lethal, for several of the animals died of doses tolerated in the case of carbon tetrachloride. The same violent contraction of sinusoids was seen after intraportal injections of chloroform as with carbon tetrachloride.

**C Dysentery (Shiga) Toxin**—0.2 c.c. of toxin containing 5 units was given subcutaneously. A moderate dilatation of the sinusoids appeared after 15 or 20 minutes and persisted for periods up to 3 hours, during which the liver was kept under observation. Twenty-four hours after toxin injection no change could be detected.

D *Diphtheria Toxin* —Similar doses of diphtheria toxin produced no visible effects

## DISCUSSION

### *Fine Anatomy of the Liver the Vascular Pattern*

Before embarking on a discussion of the controversial aspects of the liver circulation, it will be useful to remind the reader of the normal histology on which fairly general agreement has been reached

The portal vein enters the liver at the porta hepatis with the hepatic artery and bile duct, and all three soon divide into branches which spread to all parts of the liver. The vein has in its walls a generous supply of capillaries from the hepatic artery. The whole vascular bundle lies in a bed of connective tissue (Glisson's capsule) and forms the portal canals or "triads" of Kiernan, which demonstrate roughly polygonal lobules of liver parenchyma round the central hepatic veins. The hepatic venules converge to form the sublobular veins, which in turn join the collecting veins and finally flow into the main hepatic vein. Connecting the portal and hepatic radicles are the sinusoids, irregular, tortuous channels branching and anastomosing among the liver cells, but in the main taking a radial course from the portal to the hepatic veins. There is hardly any connective tissue outside the hepatic veins so that the liver cells lie close against the vessel wall. Irregular branching phagocytic cells (Kupffer cells) are distributed at intervals along the sinusoidal walls. With ordinary methods of staining all liver cells look singularly alike.

Wakim and Mann [1942 *a* and *b*] have now given good evidence for various types of arteriovenous anastomoses which were only suspected previously [Cameron and Mayes, 1930]. My studies of the transilluminated liver of living animals substantially confirms their description, but the observations on the origin and termination of the sinusoids, detailed on p. 81, have not been previously recorded and may have important bearings on the physiology and pathology of the liver.

The final distribution of the hepatic artery on which there has been such a diversity of opinion appears to have been settled by the observations of Wakim and Mann, whose findings I can fully confirm. However, I can find no support for their claim to have seen specific liver territories supplied wholly by arterial or portal blood. Direct observation, injection of vessels, and the results of vascular occlusion and Eck fistulæ all indicate that every part of the liver parenchyma receives a mixture of portal and arterial blood.

Although all authorities agree that the blood-vessels within the liver lobule present special features, the minute structure of the sinusoids and their relation to the liver cells still remain debatable despite the careful histological studies of a host of workers [His, 1860, MacGillavry, 1864, Kölliker, 1867, Henle, 1873, Kupffer, 1876, 1898, Ranvier, 1885, 1892,

Minot, 1900, Herring and Simpson, 1906, Lee, 1923, Glogengresser, 1944]

My observations (fig 16) show the sinusoids to be continuously lined by a thin transparent membrane in which the Kupffer cells occur at regular intervals. Whether they are composed of endothelial cells, are a syncytium, or are thin fibrous tubes could not be made out, but they seemed to differ from the ordinary capillaries only in size and distribution. I am unable to confirm the presence of valves seen by Kmsely. The fact that not only blood cells but minute particles of India ink measuring  $1\ \mu$  or less remained within the lumen, even when injected into the portal vein under considerable pressure, affords strong evidence that there are no stomata or gaps in the sinusoidal wall larger than these particles. Whatever its minute structure, the membrane is closely applied to but separable from the columns of liver cells, perhaps, as Trowell [1946] suggests, there is a thin protein film between the capillary wall and the liver parenchyma. I could see no Disse space or fibres connecting the sinusoids to the liver cells. Where liver cells had shrunk away or the sinusoid had collapsed, the capillary membrane was seen lifted off the liver cells, but I was unable to make out any collection of fluid, injected dye or other material in this space.

It is surprising that an organ with such a multitude of functions as the liver should possess cells so uniformly similar in all its parts. Investigators from time to time have conceived the idea of functional territories in the liver lobules, and attempted to show structural differences by more subtle histological methods. Forsgren [1918] observed acidophil granules, said to be indicative of bile salts, in the peripheral zones only. Noel [1923] found that, with an influx of glucose to the liver, glycogen granules appeared first and in largest quantity in the cells round the central vein. The distribution and activity of mitochondria were shown by Kater [1931] to have zonal characters. Cameron *et al* [1937], as a possible explanation of the zonal and focal distribution of necrotic lesions resulting from intraportal injections of carbon tetrachloride, state "It may well be that the liver is a continually changing mosaic whose pieces may vary in function and vulnerability from time to time." Mann [1942] admits the possibility of these zones of activity.

The layer of cells lying immediately adjacent to the central hepatic vein (p 87) differs in structure and arrangement from the rest of the liver cells, and also in its behaviour towards circulating dyes and toxins. Its significance will appear later when we consider the physiological and pathological applications.

### *Physiology of the Hepatic Blood-Vessels*

1 *The Distribution of Blood to the Liver Cells*—Evidence is fairly conclusive from the work of Serégy [1901], Glenard [1901], Bartlett *et al*

[1914], McIndoe and Counsellor [1927], Copher and Dick [1928], and Hahn *et al* [1945] that the flow in the large portal veins is streamlined, and that the blood from various abdominal organs regularly supplies specific territories of the liver. The effects I observed after injection of dyes either into the portal vein or into the systemic circulation confirms this conclusion. But although the dye reaches a certain part of the liver in high concentration initially, it soon mixes with the general blood-stream and passes to the rest of the liver in smaller concentration.

There is now little doubt that widespread and efficient anastomoses exist between the hepatic artery, the portal vein and sinusoids, so that the liver parenchyma receives mixed arterial and portal blood. But the relative importance of each component in the maintenance of the liver parenchyma is still undecided. Burton-Opitz [1910, 1911] found that the artery furnished 143.4 c.c. of blood per minute while the portal vein supplied 268.2 c.c. In another publication [1912] he stated that 75 per cent of the blood is from the portal vein. Barcroft and Shore [1912] estimated the arterial contribution as 39 per cent, but concluded that the liver at rest takes its oxygen essentially from the arterial supply. MacLeod and Pearce [1914] found it was 26-32 per cent. Blalock and Mason [1936] found that the artery supplied 19.5 per cent and the portal vein 80.5 per cent.

Although it is agreed that the quantity of portal blood exceeds that of arterial blood, the latter appears to be of greater importance in the maintenance of the liver cell nutrition. This is seen in the results of experiments on ligation of the hepatic artery [see discussion by Loeffler, 1928, and Cameron and Mayes, 1930]. Collected studies show that, although different animals vary in their behaviour depending chiefly on the collateral arterial supply, the exclusion of hepatic arterial blood results in the death of considerable portions of the liver. Cameron and Mayes [1930], who took special precautions to avoid the objections raised to previous work, such as infection, insufficient anatomical data, insufficient recognition of collaterals, found that ligation of the hepatic artery in the rabbit resulted in widespread necrosis.

Ligation of the portal vein, on the other hand, causes only atrophy [Solowieff, 1875, Doyon and Dufourt, 1898, Bambridge and Leathes, 1907, Rous and Larimore, 1920]. Diversion of portal blood by an Eck fistula also results in atrophy with loss of hepatic function, but sufficient function remains for maintenance of life under restricted conditions [Whipple and Hooper, 1917, Whipple, Robschey-Robins and Hawkins, 1945].

My observations show that, after an initial temporary reduction, the blood-flow in the liver returns to its original rate and volume after ligation of either the portal vein or the hepatic artery, due to the presence of abundant arteriovenous anastomoses. But the oxygen content of

the blood reaching the liver cells must necessarily change Blalock and Mason [1936] found that the hepatic arteries supplied 22-38 per cent of oxygen in five of their animals and 58-62 per cent in two McMichael [1937] showed that in the cat the liver obtained about two-thirds of its oxygen from the portal vein under normal conditions of blood-pressure But when the blood-pressure is reduced by hæmorrhage or shock, the oxygen content of the portal vein blood is reduced and the liver comes to depend more and more on the artery for its oxygen The rabbit is dependent almost entirely on the hepatic artery for its oxygen, and gets only an insignificant amount from the portal blood McMichael further believes that, apart from the amount of oxygen carried by the portal blood, the availability of this oxygen may be determined by some inherent property of the liver cells It is possible that "the hepatic artery is the dominating source of oxygen supply" [Barcroft and Shore] No doubt other nutritional factors are involved, but it is not difficult to understand the necrosis of cells which have a large part of their oxygen supply cut off, though the volume of blood reaching them is not appreciably altered The hepatic artery is the nourishing vessel of the portal vein, and its occlusion results in the degeneration of the vein wall and thrombosis, adding the effects of a portal block to the already existing arterial obstruction [Loeffler, 1936]

Within the liver the circulation shows an irregular rhythmicity which has been described by Wakim and Mann, and of which I have ample evidence Large tracts of liver are inactive for varying periods of time, the sinusoids being dilated and full of blood or contracted and nearly empty But it must not be imagined that all contracted sinusoids are inactive For instance, after the injection of adrenalin the sinusoids contract but the blood-flow through them is very active Contraction or dilatation of sinusoids appears to be one of the methods of regulating the volume of blood contained in the liver

2 *Control of Intrahepatic Circulation*—The effects of stimulating the sympathetic and parasympathetic nerves on the circulation of the liver have been deduced from the indirect evidence of changes in liver volume, changes in hepatic inflow and outflow and alterations in portal pressure Bayliss and Starling [1894 *a* and *b*] found a rise in portal pressure on electrical stimulation of the splanchnic nerves of dogs François-Frank and Hallion [1896] obtained a reduction in the volume of the liver and a rise in portal pressure on stimulating the splanchnic nerves of the dog Schmid [1909] noted a decrease of inflow through the portal veins and a rise of portal pressure on stimulating the hepatic plexus Burton-Opitz [1914] concluded that the hepatic plexus supplied vasomotor fibres to the portal radicles Neubauer [1913] found that the volume of the liver increased on stimulating the central end of the cut vagus or splanchnics while it decreased when the peripheral end of the vagus was stimulated MacLeod and Pearce [1914] noted that

stimulation of the hepatic plexus caused constriction of the hepatic artery and to a lesser extent the portal vein. Edmunds [1914-15] obtained an increase in hepatic volume on stimulating the splanchnics, which he attributed to constriction of the sublobular veins. Griffith and Emery [1930] found a shrinkage of the liver on sympathetic stimulation but stimulation of the vagus had no effect. Bauer, Dale, Poulsson and Richards [1932] obtained similar results, but noted also that portal inflow and the outflow from the liver both increased on sympathetic stimulation. McMichael [1937] found no evidence of parasympathetic action on the liver vessels. Wakim [1942], from direct observations on living animals, came to the conclusion that stimulation of the sympathetic plexus caused constriction of the intrahepatic vessels, while stimulation of the vagus had no perceptible effect.

My observations agree with the findings of the last workers, but the blood-flow appeared to be accelerated in the constricted vessels. The whole appearance was one of increased activity, and may explain the reduction in volume with increased outflow and increased portal pressure obtained by some of the experimenters. The liver therefore appears to receive vasoconstrictor fibres from the sympathetic but no vasodilators from the parasympathetic.

It has been demonstrated by many workers that adrenalin decreases the liver volume by constricting the hepatic arterioles, portal radicles and sinusoids [Burton-Opitz, 1912, MacLeod and Pearce, 1914, Lamson and Roca, 1921, Loeffler and Nordmann, 1925, who made direct observations of transilluminated livers, Lampe and Mehes, 1926, Clark, 1928]. The majority of them also noted a rise of portal pressure and some an increased outflow. Bauer, Dale, Poulsson and Richards [1932] found that in dogs, adrenalin constricted ramifications of both hepatic artery and portal vein, whilst opening the "sluice" mechanism located near the opening of the larger hepatic veins into the inferior vena cava. McMichael [1932] suggested that the decrease in the liver volume as measured by a plethysmograph indicated that the constrictor effect must be on the portal venous ramifications and not on the sublobular and larger hepatic veins. Tschernogoroff and Popoff [1936] also noted a decrease in liver volume and a rise of portal pressure in dogs. From perfusion experiments Chakravarti and Tripod [1940] concluded that all sympathetic drugs contracted the portal radicles and hepatic arterial branches, which increased the resistance to inflow and raised the portal pressure, at the same time they opened the "sluice mechanism," thus increasing the outflow, draining the liver of blood and reducing its volume.

My experiments suggest a marked constriction of all the ramifications of the portal radicles, sinusoids and hepatic arterioles with adrenalin. Although I was unable to identify a "sluice" mechanism, the extreme activity and rapidity of flow in the narrow sinusoids indicated that

resistance to the outflow had been reduced, which may well be the opening of the "sluice"

The evidence is fairly conclusive that sympathetic nerves and certain internal secretions like adrenalin can influence the hepatic circulation, but it is not quite clear under what circumstances these mechanisms act. The sympathico-adrenal system is called upon as an emergency measure when the body has to cope with some sudden emotional or physical stress [Cannon, 1932]. Among other effects it causes acceleration of the heart, faster blood-flow, a rise of blood-pressure, splanchnic vasoconstriction and a redistribution of blood. These must certainly reflect on the liver circulation in addition to any direct sympathetic and adrenal effects. It is perhaps under such conditions of stress and pain that sympathetic and adrenal control of the liver circulation is called upon. In my experiments a wide variety of stimuli—*e.g.* trauma, burns, heat and cold, and mechanical stimuli—caused a temporary contraction of sinusoids before their more specific effects became apparent. The one common property of all these stimuli is that in the unanaesthetised animal they would be painful. It seems therefore fair to suggest that painful stimuli called forth the sympathico-adrenal response, the effects of which on the liver are vasoconstriction with increased rate of flow.

The action of acetylcholine on the liver circulation has been investigated with a view to estimating parasympathetic influence. Hunt [1918] observed a diminution in liver volume with small doses, which he ascribed to diminished inflow during the general vasodilatation. With larger doses in dogs he found this diminution followed by an increase in volume, which he thought was due to increased inflow through a dilated hepatic artery when the general effect was passing off. Bauer, Dale, Poulsson and Richards [1932] could find no marked effect in the dog even with large doses. There was, however, a tendency to relaxed arterial tone, retardation of portal flow, increase of liver volume and decreased outflow. McMichael [1933], in a series of carefully controlled experiments in the cat, demonstrated a constrictor effect on the portal vein with large doses. With smaller doses he noted an initial fall in portal pressure due to diminished inflow into the portal system as a result of general vasodilatation and diminished cardiac output followed by a rise due to increase of general venous pressure during the cardiac slowing. He found no evidence of any parasympathetic dilator action on hepatic vessels. The contraction with large doses was, he thought, a direct effect on the vessel wall.

In my experiments acetylcholine, after a momentary contraction, produced dilatation of sinusoids and portal ramifications with stasis of blood. The appearances suggested that the vasodilatation and retardation were effects of the peripheral vasodilatation and cardiac slowing. The fact that with local application the constriction was immediate,



but dilatation was no more rapid than by injection into the portal or systemic circulation, indicates a direct constrictor effect as suggested by McMichael, followed by a delayed dilator effect resulting from extra-hepatic vascular changes. It is clear that the liver blood-vessels are susceptible to acetylcholine. But stimulation of the vagus results in no vascular response. One must, therefore, infer that vagus fibres do not reach the hepatic blood-vessels.

Mautner and Pick [1915, 1922, 1931 *a* and *b*] and Lampe and Mehes [1926] demonstrated the "sluice" mechanism on which histamine and other "shock poisons" have a powerful constrictor effect. According to Bauer, Dale, Poulsson and Richards [1932] the site of action of histamine was in the neighbourhood of the caval entries. Dale and Richards [1918] and Dale and Laidlaw [1919] thought that the peripheral capillary dilatation and the effects on the general circulation were the dominant factors.

The very rapid dilatation I noticed with histamine or light trauma, such as stroking the liver surface, which presumably liberated histamine-like substances, appeared to be a direct effect on the sinusoidal wall. The reduction in the rate of flow appeared to be the result of widespread capillary dilatation, although the closing of a sluice mechanism cannot be altogether denied.

A third factor which undoubtedly influences intrahepatic blood-flow is respiration. The portal pressure rises during inspiration and falls during expiration. Schmid [1909] attributed these variations to changes in intra-abdominal pressure. McMichael [1932], however, points out that the fluctuations are present even when the abdomen is open and therefore intra-abdominal pressure cannot be entirely responsible. He suggests that the factor responsible is the squeezing effect of the descending diaphragm. Franklin and Janker [1937] also noted emptying of the liver vessels during inspiration and concluded that the main hepatic outflow was during inspiration. My experiments indicate a general constriction and emptying of the sinusoids during deep breathing with an increase in the constriction during inspiration in each respiratory cycle. The squeezing action of the diaphragm no doubt plays a part. But it is well recognised that the increase of negative pressure during inspiration has an aspirating effect on the great veins in the thorax. It is conceivable that this effect is communicated to the hepatic veins which open directly into the inferior vena cava quite close to the heart, provided no sphincteric action intervenes at the ostia of the hepatic vein.

### *Relation of Hepatic Circulation to Hepatic Pathology*

The peculiar distribution and connections, the high reactivity, and the very abundance of the liver vessels must play a large part in the pathogenesis of lesions in the liver.

The part played by environment and climate in the production and course of pathological processes is being increasingly realised "Chill" has been vaguely spoken of as a predisposing cause of many diseases Heat cramp, heat exhaustion and heat stroke are recognised clinical entities The therapeutic use of ice-bags, hot-water bottles, plasters, etc is based on the belief that such measures could influence the circulation of deeper structures There is experimental evidence to prove that heat and cold applied to the body surface can modify the circulation of internal organs Kuntz and Haselwood [1940] demonstrated that moderate cooling of the skin produced vasoconstriction in the viscera of the segmental area while moderate warmth caused vasodilatation

My observations show that the liver vessels react readily by contraction to cooling the skin and by dilatation to warming Such effects, if prolonged or repeated, must certainly modify the function of liver cells Even if they are not severe enough to cause gross structural changes, it is reasonable to suggest that they render the liver parenchyma more susceptible to attacks by injurious agents Climate may be one of the factors contributing to the frequency of liver lesions, *e.g.* cirrhosis, in the tropics Apart from direct injury, bacterial toxins or products of protein breakdown, by the fever they produce, may cause vasodilatation and stagnation in the liver circulation, upsetting its nutrition, impairing its function, and thus contributing to the general clinical picture

In traumatic shock the dilatation of the vast vascular bed, which I have described in animals with crushed limbs, must remove a fair proportion of blood from effective circulation, and its retardation must certainly result in anoxia and nutritional changes in the liver cells Engel, Harrison and Long [1944] produced evidence to show that the rise of blood amino-acid nitrogen in shock was due either to a failure of the sluggish liver circulation to pass enough amino-acids through the liver, or to liver-cell damage by anoxia which rendered them incapable of dealing with the circulating amino acids Frank, Seligman and Fine [1946] showed that loss of liver integrity was a significant factor in the collapse of shock, and that maintaining, artificially, the liver circulation prevented the grave effects of shock in animals The actual dilatation of the sinusoids is probably produced, like that of capillaries elsewhere, by histamine-like substances liberated at the site of trauma The suggestion that such substances shut the sluice mechanism at the exit of the hepatic veins has already been discussed Before dilatation of vessels, the shock-producing as well as other painful stimuli *e.g.* burns, heat and cold, etc, produced a temporary contraction This would appear to be the sympathico-adrenal response of Cannon, which is now a well-recognised phenomenon seen as an emergency protective measure against emotional and physical stresses

In my transillumination experiments hæmorrhage was seen to produce marked narrowing of the liver vessels. Pilcher and Sollman [1914] showed that blood-vessels of organs responded to hæmorrhage by contraction, which they thought was effected through the vasomotor nerves. According to Cannon [1932], hæmorrhage resulted in contraction of blood-vessels through the operation of the sympathico-adrenal response. Long [1947] mentions hæmorrhage as one of the factors that releases adrenalin. My observations are in conformity. Such narrowing of vessels and retardation of blood-flow in the liver occurred, that derangement of hepatic function must certainly be expected to result. Ireneus and Puestow [1934] found that after massive hæmorrhage in dogs there was a reduction in the ability to assimilate galactose and a prolongation of the prothrombin time.

There is experimental evidence that alterations in blood-volume influence blood-flow in internal organs, including the liver. Forbes and Nason [1935] injected hypertonic saline and urea and found by direct observation two types of reaction in the cerebral vessels of the cat: an immediate dilatation of the arteries and arterioles due to a direct action, and a delayed dilatation of venules probably due to a shift of fluid from the subarachnoid space to the blood-stream. Earlier, Bayliss and Starling [1894] had demonstrated that the portal system took up a large portion of the extra volume resulting from intravenous injection of hypertonic solutions. With intraportal injection of hypertonic solutions I observed a dual response of the liver vessels: an immediate contraction of sinusoids, due probably to a direct action, followed by a delayed dilatation, probably resulting from the increase in blood-volume due to withdrawal of fluid from the tissue spaces into the blood-stream.

An experimental procedure which is of interest and has its parallel in human pathology is the obstruction of the hepatic veins. Chronic venous congestion or "nutmeg liver" is a familiar pathological finding. The effect of obstructing the hepatic vein has been studied experimentally by Bolton [1914], Jacobson and Goodpasture [1918], Simonds and Brandes [1925], Simonds and Calloway [1932], Simonds and Jergesen [1935], Bolton and Barnard [1931] and Kelsey and Comfort [1945]. They all found marked congestion and enlargement of the liver, dilatation of all its vessels, and central necrosis of the liver lobules. My observations agree with these findings, showing a great and rapidly developing dilatation of the sinusoids and other vessels in all parts of the liver, which is the result to be expected when the only outflow from the liver is obstructed. But what is of great interest is the localisation of the necrosis to the cells round the central vein of the liver lobules. It is now generally agreed that the necrosis is not due to pressure. In my experiments, vessels and sinusoids in all parts of the liver were equally dilated. The central zones showed no difference. If pressure

were responsible, the peripheral zones where it is highest should suffer most. Anoxia is suggested as a cause and is accepted by most authorities. Blalock and Mason [1936] demonstrated a rapid fall in oxygen tension within the liver lobule from the periphery to the centre. The hepatic vein blood contained much less oxygen than blood from other veins. It is clear that with a retarded blood-flow the peripheral cells have greater opportunities for removing oxygen, reducing further the oxygen tension in the blood reaching the centrilobular zones. Without minimising the importance of the above factor, I wish to draw attention to the structural and functional differences (as shown by behaviour to circulating dyes) which I have described in the centrilobular cells, and to offer as an additional factor Mallory's [1914] suggestion that the greater vulnerability of the centrilobular cells is due to their greater functional activity and higher degree of specialisation.

Severe anoxia is known to be a potent cause of capillary damage, and the liver sinusoids appear to afford no exception. Campbell [1928] exposed rabbits, guinea-pigs, rats and mice to very low oxygen tensions, and found engorgement of the liver with dilatation of the sinusoids. He described the liver-cell columns as merely threads in a network, and a drawing of a mouse liver shows enormously distended sinusoids. He suggested two possible causes: plethora following polycythaemia due to anoxia, and liberation of histamine-like substances. Rich [1930] placed rats in low oxygen tension chambers and showed diminished ability to excrete intravenously injected bilirubin and changes in the liver cells. Hurtado *et al* [1945] suggested insufficiency of the liver in excretion of pigment to explain the hyperbilirubinæmia observed at high altitudes. My observations suggest that the dilatation was too rapid to be accounted for by plethora. I have no evidence to confirm or deny the possibility of histamine-like substances being liberated. Whatever the cause, anoxia results in damage to the sinusoids, leading to marked dilatation of sinusoids and retardation of blood-flow which must certainly affect the nutrition of the liver parenchyma and derange its function.

In investigations on the pathogenesis of hepatic cirrhosis, the effects of toxic agents on the liver have been extensively studied. Chloroform, alcohol, tar, carbon tetrachloride, phosphorus, manganese, arsenic, copper, bacteria and their products are some of the agents used. Moon [1934] reviews a very complete list. The literature on carbon tetrachloride, the most studied substance, is fully reviewed by Cameron and Karunaratne [1936], who describe in detail the histological changes produced in the liver. After a single dose they found little evidence of change until between 5-24 hours. Degenerative changes, accompanied by marked congestion, then appeared in the central and mid zonal regions. Wakim and Mann [1942b] observed in transilluminated rat livers a momentary constriction of sinusoids with quick recovery when

carbon tetrachloride was inhaled. With longer exposures or subcutaneous injections they found degenerative changes in the liver cells and obliteration of sinusoids, but they describe no precise distribution, and it is the peculiar centrilobular distribution which needs explanation. Glynn and Himsworth [1948] studied the intrahepatic circulation after carbon tetrachloride by injection of mandarin black into the portal circulation through the spleen, and concluded that the circulatory impairment due to compression of sinusoids by swollen liver cells in the periphery of the lobule accounted for the central necrosis. A direct constrictor effect on the sinusoids was discounted, because similar changes were found in necrosis due to dietetic insufficiencies and in vascular occlusions, neither of which cause spasm of sinusoids. Andrews and Maeraith [1948], also using India ink, came to a similar conclusion but found constriction of sinusoids in the mid-zonal region. Their microphotographs show patent and even dilated sinusoids in the periphery of the lobule 8 and 24 hours after carbon tetrachloride. They refer to the constant findings of congestion by Cameron and Karunaratne, and admit that more than one mechanism may lead to centrilobular anoxia.

The series of changes in the livers of rats which I have observed suggest a different mechanism. The initial constriction of vessels appears to be the common response of the sinusoids to any irritant, for it occurs on rapidly injecting hypertonic saline, 40 per cent urea, cold normal saline or 50 per cent glucose directly into the portal vein. After carbon tetrachloride, dilatation soon follows and persists. In 6-8 hours degenerative changes are seen in the cells of the central areas, while the sinusoids in all parts of the lobule are still dilated and very active. Dyes and India ink injected at this time reach all parts of the lobule readily. The degenerative changes progress and ultimately compress and disorganise the sinusoidal pattern in the centrilobular zones. I wish to emphasise that the central cells degenerate first and disorganisation of the circulation follows as a direct result. The central necrosis appears to be the result of direct action of carbon tetrachloride, although it has been argued that the cells first in contact with the substance should show most damage. I offer three possible explanations suggested by structural and functional differences in the central zones to which I have alluded before.

- (i) The centrilobular cells abstract and concentrate dyes. It is reasonable to believe that they do the same to toxic agents.
- (ii) They are more vulnerable owing to greater functional activity and to structural differences.
- (iii) Rous, Gilding and Smith [1930] demonstrated a gradient of permeability in the capillaries of muscles increasing from the arterial to the venous end. The greatest permeability was at the junction of the capillary with the vein. Dyes circulating

in the liver appear in greatest concentration outside the central sinusoids and their junctions with the central hepatic vein, a position analogous to the capillary venous junction in muscles, suggesting a similar permeability gradient which will allow toxic material to leak out in maximum quantities in the central zones

The frequent occurrence of secondary malignant deposits and pyæmic abscesses is evidence of the readiness with which emboli are arrested in the liver circulation. On the other hand, the rarity of infarcts from embolism is well known. Complete lists of the cases reported in the literature, which are far from numerous, reviewed by Zimmerman [1930], Lund, Stewart and Lieber [1935] and Pass [1935], show that many of them are not true infarcts but focal necroses resulting from trauma, infection or toxins. Frequent collateral arterial supplies, numerous arteriovenous anastomoses, and complex sinusoidal connections render it almost impossible to cut off the blood-supply to the liver cells by occluding a short segment of any intrahepatic vessel. The effect of circulating starch granules bears out this statement. Although they are arrested at numerous points in the vessels, blood-flow is not affected, the points of obstruction being easily by-passed. A considerable length of portal vein with its branches and arteriovenous connections must be occluded before an embolus can shut off the blood-supply to the liver cells.

### SUMMARY AND CONCLUSIONS

The circulation in the intact liver and its reaction to various stimuli were studied in frogs, mice and rats by a modification of Knusely's transillumination technique.

Characteristic patterns in the anatomy and distribution of the portal and hepatic veins are described. Sinusoids appear as thin-walled collapsible tubes with complete linings. Arteriovenous anastomoses are frequent, and reconcile the rarity of infarcts from embolism with the frequency of pyæmic abscesses and metastatic deposits. The behaviour of circulating dyes and toxic agents suggest the presence of functional zones in the liver lobule. Certain structural differences, too, are noticed in the centrilobular areas.

Under normal conditions the liver circulation shows an irregularly intermittent rhythmicity. Blood-flow is under sympathetic control, but there is no evidence of a parasympathetic supply. A chemical control is suggested by the effects of hormones such as adrenalin, acetylcholine and histamine, while respiration has a very definite influence.

Stimuli such as obstruction of the vessels, mechanical trauma, hæmorrhage, heat and cold induce rapid responses in the liver circula-

tion Indeed the centrally important observation is the readiness with which it reacts to stimuli, and this may have a significant bearing on the pathogenesis of liver diseases

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THE DETERMINATION OF THE RENAL CLEARANCE OF  
INULIN IN MAN By J S ROBSON, M H FERGUSON,  
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INTRODUCTION

HOMER SMITH and his colleagues [1937] have claimed that the measurement of the renal clearance of inulin is a valid method for the determination of the rate of glomerular filtration. When applied to human subjects, the difficulties of the procedure [Smith, Goldring and Chasis, 1938], which involves continuous infusion of inulin and the collection of carefully timed and accurately measured samples of urine, have militated against the general use of the method. Hence simpler though less precise tests of renal function have retained their popularity in clinical work.

Many attempts have been made to simplify the measurement of inulin clearance, an indication at once of its potential value and its difficulty. Alving and Miller [1940] assumed that about sixty minutes after a single intravenous injection of inulin, equilibrium was attained between the concentration in the plasma and that in the extravascular fluid, the plasma concentration thereafter decreasing exponentially with time, as a result of the renal excretion of inulin. They collected urine over measured intervals and calculated the mean values of the plasma-inulin concentration for these intervals, by interpolation, from the curves relating this quantity to time. No material simplification was introduced by Findley and White [1940], who injected inulin subcutaneously instead of by the intravenous route, the necessity for catheterisation remained. Nor was much real help obtained from the suggestion of Earle and Berliner [1946] that a sustaining infusion of inulin given at such a rate as to maintain a constant plasma-inulin concentration would itself measure the rate of loss of inulin from the urine. Although this procedure would dispense with the necessity for collecting and measuring urine, it demands, *inter alia*, prior knowledge of the rate to be measured, since inulin cannot be determined in plasma sufficiently rapidly for the infusion rate to be adjusted and readjusted

in the course of a single experiment Furthermore, the method involves the assumption that constancy in the plasma concentration of inulin necessarily implies constancy in the total amount of inulin in the body, variations in the amount of inulin in the extravascular compartment, however, cannot be detected Landowne and Alving [1946] have proposed a method by which, from a single injection of para-amino-hippuric acid, calculation yields figures representing the glomerular filtration rate, renal plasma flow and maximal tubular excretory capacity, this method again demands the collection of urine by catheter over short periods of time

The use by Barnett [1940] of the single injection method, and the calculation of clearance from the slope of the falling curve obtained by plotting plasma inulin concentration against time on semi-logarithmic paper, was an attempt to evade the major technical difficulty of accurate collection of urine by catheter The validity of the method is dependent upon the truth of the assumption that the intravenous injection of inulin is followed by a period in which the plasma level rapidly falls until equilibrium between plasma and extravascular body fluid is attained, and that thereafter the plasma concentration is a simple exponential function of time It will be shown that this assumption is unjustified, but that it is, nevertheless, possible to measure accurately the renal clearance of inulin without catheterisation

## PART I VOLUME OF DISTRIBUTION

### *Theoretical Considerations*

When inulin is introduced into the blood-stream, part passes out of the blood-vessels and is distributed in the body fluids It is established that inulin is neither destroyed nor stored in the body [Smith, 1937] Kruhoffer [1946] has shown that it does not enter red blood cells or liver cells, nor is it fixed by the reticulo endothelial system He presents evidence that it becomes distributed in a volume approximately equal to the extracellular space It is excreted from the body solely by the kidneys at a rate which is reported to be proportional to the plasma level [Shannon and Smith, 1935, Miller, Alving and Rubin, 1940]

Dominguez [1934, 1935] claimed that when creatinine was injected into the blood-stream it became evenly distributed throughout a "volume of distribution," and that equilibrium between vascular and extravascular fluids was then maintained in the face of continued excretion of creatinine by the kidneys The same conception was applied to galactose [Dominguez and Pomerene, 1944] and was invoked in the calculation of the renal clearance of mannitol and inulin by Newman, Bordley and Wintermütz [1944], Barnett [1940], Alving and Miller (1940), and more recently for mannitol by Dominguez, Corcoran and Page [1947]

The volume of distribution as defined and used by these workers is simply that volume which would contain all the solute present in the body if it were evenly distributed at the concentration found in the plasma. This may be expressed algebraically thus

$$V_x = \frac{X_b}{P}, \quad (1)$$

where  $V_x$  = the volume of distribution of the substance under consideration

$X_b$  = the total amount of the substance in the body

$P$  = the concentration of the substance in plasma water

It is clear that if the concentration of solute is in fact uniform throughout the fluids which contain it, the function  $V_x$  has a real significance, for certain solutes, for example, it appears to equal the plasma volume plus the volume of extravascular extracellular fluid. If the concentration of the solute is not uniform throughout these fluids, the value of the function  $V_x$  will bear no obvious relation to the volume of any actual fluid compartment. Whichever of these possibilities represents the true state of affairs in a given case is immaterial to the concept of the volume of distribution. In either case, the volume of distribution as defined will be constant so long as equilibrium is maintained between the vascular and extravascular fluids but it cannot be constant otherwise.

For inulin, equation (1) may be written

$$V_I = \frac{I_b}{P}, \quad (2)$$

where  $V_I$  = the volume of distribution of inulin

$I_b$  = the total amount of inulin in the body

$P$  = the concentration of inulin in plasma water

The value of the function  $V_I = \frac{I_b}{P}$ , at a time when equilibrium does not exist, provides a measure of the difference between the concentration of inulin in plasma water and the mean concentration in the volume of the extravascular compartment throughout which inulin is distributed, since it may be assumed that the volume of plasma water remains constant and the volume of distribution during the time of equilibrium has a unique value. Moreover, the rate at which the function  $V_I$  changes, following the intravenous injection of inulin, provides a value for the change in the amount of inulin in the extravascular compartment relative to the plasma-water concentration, with respect to time. These relationships may be seen from the following analysis —

Let  $I_e$  = the total amount of inulin in the extravascular compartment of the volume of distribution

$E$  = the mean concentration of inulin in the extravascular compartment of the volume of distribution

$V_e$  = the volume of the extravascular compartment throughout which inulin is distributed

$V_p$  = the volume of the plasma water

$I_p$  = the total amount of inulin in the plasma

Then

$$V_I = \frac{I_b}{P} = \frac{I_p + I_e}{P}$$

By definition,

$$I_p = P \times V_p$$

$$\begin{aligned} V_I &= \frac{V_p P}{P} + \frac{I_e}{P} \\ &= V_p + \frac{V_e E}{P} \end{aligned}$$

Differentiating with respect to time,

$$\begin{aligned} \frac{dV_I}{dt} &= \frac{dV_p}{dt} + V_e \frac{dE}{dt} \\ &= V_e \frac{dE}{dt} \end{aligned}$$

## METHODS I

### *Determination of the Function $V_I = \frac{I_b}{P}$*

The determination of the values of the function  $V_I = \frac{I_b}{P}$  at intervals following the intravenous injection of inulin was performed on nine subjects ranging in age from 25 to 78 years. Five were old people suffering from varying degrees of hypertension but who otherwise had no complaint. The remaining four were normal volunteers. With the exception of the two female volunteers, who were permitted to be sedentary, the subjects were recumbent during the period of the experiment.

All subjects were prepared by fasting overnight, no breakfast was given and they were urged to drink ample fluid. Accurately measured amounts of inulin, dissolved either in normal saline or in 0.5 per cent gum acacia in normal saline, were given intravenously. Each injection

took approximately five minutes, and the time of the injection was reckoned as the mid-point of that period. Venous blood was removed at intervals of about twenty minutes for two to three hours after injection. Except from the two normal female volunteers, urine was collected by catheter over periods of approximately twenty minutes for a similar length of time, the bladder was washed out with 20 ml of normal saline before each collection, the exact time of all procedures was noted. The female volunteers passed urine spontaneously at twenty-minute intervals, and errors in collection were minimised by establishing urine flows of over 10 ml/minute during the period of the experiment. Inulin was determined in all urine and plasma samples by Cole's unpublished modification of Steinitz's method [1938], in which inulin is hydrolysed to fructose, the Selwanoff reaction being then applied and the colour read in a Hilger photoelectric colorimeter using an Ilford colour filter No. 302.

The total inulin in the body at each time of urine collection was derived by subtraction of the amount excreted from the amount injected. The values of  $P$  employed were derived from the plasma inulin-time curve at the times of urine collection. They were converted to mg inulin/100 ml plasma water, using the formula  $\text{total plasma water}/100 \text{ ml plasma} = 100 - \text{plasma proteins in g}/100 \text{ ml plasma}$  [Goldring and Chasis, 1944]. The values for  $V_I$  so obtained, expressed as litres, were plotted against time on simple graph paper.

## RESULTS I

Table I presents the clinical features of the subjects and the results of the values for  $V_I = \frac{I_b}{P}$  calculated at intervals following the injection of inulin. In all subjects the function  $V_I$  shows an increase with time throughout the period of the experiment. The initial values, calculated from 16 to 38 minutes after injection, range from 7.2 to 13.9 l. The final values, calculated at times varying from 68 to 150 minutes following injection, range from 12.1 to 28.4 l. Increasing the dose of inulin from 49.7 mg to 217.0 mg per kg body-weight, as in case No. 1 (J.R.), has little effect on the value for  $V_I$  20 to 40 minutes after injection, but produces a considerable increase in  $V_I$  later.

Fig. 1 for cases 2-9 and fig. 2 for case 1 (J.R.) illustrate graphically the change of  $V_I$  with respect to time. Each line is drawn between the values of  $V_I$  calculated respectively 20 to 40 minutes and 100 to 120 minutes after injection, without consideration of the intermediate points which, however, fall close to the line. In case 2 (G.S.) the final value for  $V_I$  was calculated only 68 minutes following injection.

The results clearly indicate that at no time during the period of the experiments did the function  $V_I$  attain a constant value. Following



TABLE I—*Continued*

Subject	Sex	Age, years	Weight, kg	B P, mm Hg	Inulin dose, mg/kg	Time in mins following injection	$V_I = \frac{I_b}{P}$ litres
8 A A	M	78	77.7	200/100	101.9	30	13.9
						51	18.0
						71	21.8
						103	28.4
9 J O	M	75	60.5	230/115	98.1	20	8.5
						40	10.2
						62	11.2
						81	11.8
						101	12.5
						121	13.1

the intravenous injection of inulin, therefore, equilibration of distribution between the plasma and the extravascular fluids did not occur. The

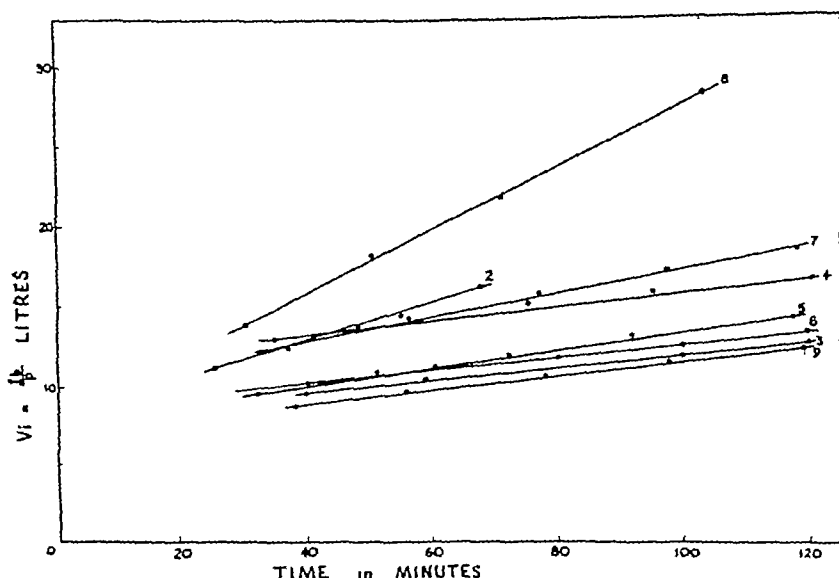


FIG. 1—Values of the function  $V_I$  plotted against time on simple graph paper for subjects Nos 2-9. The numbers shown correspond to the subjects as listed in the tables. The line drawn in each case is that between the value of  $V_I$  calculated between 20 and 40 minutes following injection and the value calculated 100 to 120 minutes following injection, without consideration of intermediate points.

implications of this conclusion on attempts to derive the renal clearance of inulin from the slope of the plasma inulin-time curve following intravenous injection are discussed in the theoretical considerations which follow.

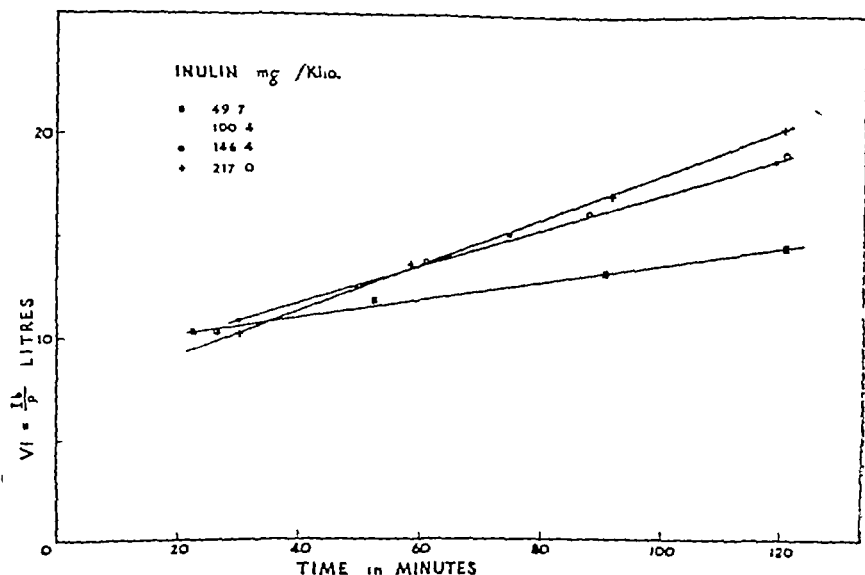


FIG 2—Values of the function  $V_I$  plotted against time on simple graph paper for four experiments performed on subject No. 1 (J.R.) The symbols indicate the dose of inulin in each experiment. The lines drawn are those between the values of  $V_I$ , calculated 20 to 40 minutes and 100 to 120 minutes following injection, without regard to intermediate points. No line is drawn between the points derived in the experiment using inulin given in the dose 146.4 mg/kg body weight.

## PART II RENAL CLEARANCE

### *Further Theoretical Considerations*

Inulin disappears from the body at a rate determined only by the renal excretion [Smith, 1937]. This may be expressed

$$\frac{dI_b}{dt} = -uv, \quad (3)$$

where  $uv$  equals the rate of excretion of inulin in the urine.

The negative sign indicates that the change in the body is a loss.

By definition,

$$\begin{aligned} I_b &= P \times V_I \\ \frac{dI_b}{dt} &= \frac{d}{dt}(P \times V_I) \\ &= P \frac{dV_I}{dt} + V_I \frac{dP}{dt} \end{aligned}$$

By formula (3),

$$\begin{aligned} -uv &= P \frac{dV_I}{dt} + V_I \frac{dP}{dt} \\ -\frac{uv}{P} &= \frac{dV_I}{dt} + \frac{V_I}{P} \frac{dP}{dt} \end{aligned}$$



$\frac{uv}{P}$  represents the clearance in terms of plasma water. Assuming its constancy and representing it by  $C$ , we have

$$\frac{V_I}{P} \frac{dP}{dt} = - \left( C + \frac{dV_I}{dt} \right) \quad (4)$$

If, following the injection of inulin, equilibration between plasma and the extravascular component of the volume of distribution were attained and maintained, the function  $\frac{dV_I}{dt}$  would become zero since  $V_I$  would be a constant. Under these circumstances formula (4) becomes

$$V_I \frac{dP}{dt} = -CP \quad (5)$$

This is the formula established by Newman, Bordley and Winternitz [1944] for mannitol and stated by them to be applicable to inulin. Its validity depends upon its being restricted to the circumstances governing its derivation, i.e. upon the establishment and maintenance of equilibrium between the compartments of the volume of distribution. Indeed, it is only in these circumstances that the plasma concentration of any substance not metabolised, and excreted by a renal mechanism at a rate proportional to the plasma concentration, is exponentially related to time.

The error introduced by the application of formula (5) to circumstances in which equilibration following injection is not maintained may be derived as follows —

Rearranging formula (5) and substituting  $C'$  for the value of clearance obtained by its use and  $V_I'$  for the volume of distribution at equilibrium, we have

$$C' = - \frac{V_I'}{P} \frac{dP}{dt}$$

By formula (4),

$$C = - \frac{V_I}{P} \frac{dP}{dt} - \frac{dV_I}{dt}$$

The error in plasma water clearance between these formulæ is

$$\begin{aligned} C - C' &= \frac{V_I'}{P} \frac{dP}{dt} - \frac{V_I}{P} \frac{dP}{dt} - \frac{dV_I}{dt} \\ &= \frac{V_I' - V_I}{P} \frac{dP}{dt} - \frac{dV_I}{dt} \end{aligned}$$

It is seen that the error is not constant with time

At time  $t$ , such that  $V_I = V_I'$  (i.e. when  $E$  is directly proportional to  $P$ ),

$$C - C' = -\frac{dV_I}{dt}$$

At any other time the error increases or decreases according to whether  $V_I$  is greater or smaller than  $V_I'$ , i.e. according to whether  $P$  is smaller or greater than the mean concentration of inulin in the extravascular compartment of the volume of distribution

For the purpose of solving equation (4), the simplifying assumption is made that between definable limits of time and inulin dose the function  $V_I = \frac{I_b}{P}$  is linearly related to time. Figs 1 and 2 suggest that this assumption is justified as an approximation, but the magnitude of the error introduced will be discussed later. For the purpose of further analysis, therefore, the function  $V_I$  can be expressed as a line,

$$V_I = a + bt,$$

where  $a$  and  $b$  are constants of the line

Formula (4) then becomes

$$\frac{a + bt}{P} \frac{dP}{dt} = -(C + b)$$

Solving this differential equation for  $P$ , we have

$$\int \frac{dP}{P} = -(C + b) \int \frac{dt}{a + bt}$$

$$\ln P^* = -\frac{(C + b)}{b} \ln(a + bt) + \ln K,$$

where  $\ln K$  is the constant of integration

$$\ln P = \ln K(a + bt)^{-\frac{(C+b)}{b}}$$

$$P = K(a + bt)^{-\frac{(C+b)}{b}} \quad (6)$$

or

$$P = K V_I^{-\frac{(C+b)}{b}} \quad (7)$$

It is clear that equation (7) may be solved for  $C$ , given two plasma water inulin values  $P_1$  and  $P_2$ , determined at times  $t_1$  and  $t_2$ , within the limits of time during which it may be assumed that  $V_I$  is linear

\* "ln" = natural logarithm

Let  $(V_I)_1$  and  $(V_I)_2$  equal the function  $\frac{I_b}{P}$  at times  $t_1$  and  $t_2$ , we have

$$P_1 = K(V_I)_1^{-\frac{(C+b)}{b}}$$

and

$$P_2 = K(V_I)_2^{-\frac{(C+b)}{b}}$$

$$\log P_1 - \log P_2 = -\frac{(C+b)}{b} [\log (V_I)_1 - \log (V_I)_2] *$$

$$C = \frac{b(\log P_1 - \log P_2)}{\log (V_I)_2 - \log (V_I)_1} - b \quad (8)$$

The calculative error in the determination of renal clearance employing formula (8) is solely the error introduced by the assumption of linearity in the function  $V_I$ . The error is represented by deviations of the values of  $P$  determined by the use of formula (6) from those derived from the plasma inulin-time curve constructed from experimentally determined plasma levels. For this purpose formula (6) can be rearranged

$$P = K(a + bt)^{-\frac{(C+b)}{b}} \quad (6)$$

$$P = Ka^{-\frac{(C+b)}{b}} \left(1 + \frac{bt}{a}\right)^{-\frac{(C+b)}{b}}$$

Put

$$P_0 = Ka^{-\frac{(C+b)}{b}}$$

$$P = P_0 \left(1 + \frac{bt}{a}\right)^{-\frac{(C+b)}{b}} \quad (9)$$

$P_0$  is the value of  $P$  corresponding to the time  $t=0$

The method of application and the results obtained by the use of formulæ (8) and (9) with data derived from experiments performed on human subjects will be discussed in the next section

## METHODS II

### *The Experimental Determination of the Renal Clearance of Inulin*

The renal clearance of inulin was determined on nine subjects, using the data obtained in the experimental determination of the function

$V_I = \frac{I_b}{P}$  and utilised in constructing Table I. Values for renal clearance

following the intravenous injection of inulin were obtained during two to six periods of approximately 20 minutes each, by dividing the mean

\* "log" = logarithm to the base 10

rate of inulin excretion per minute by the plasma level, the latter being read from the curve of plasma-inulin values plotted against time. The concentration selected was the one occurring  $2\frac{1}{2}$  minutes before the mid-point of the clearance period\*. Although urine was collected approximately every 20 minutes after injection, no clearance value was calculated before 30 minutes following injection, the phase of rapidly falling plasma inulin being thus avoided. No clearance was calculated after the plasma inulin had fallen below 15 mg/100 ml plasma. The values obtained in this way will be termed "experimentally determined" clearances.

### *The Determination of the Renal Clearance of Inulin Employing Formula 8*

Between the times  $t_1$  and  $t_2$ , stated in each case, and approximately 30 and 120 minutes respectively after injection, the curves of the function  $V_I = \frac{I_b}{P}$  were assumed to be linearly related to time. The times  $t_1$  and  $t_2$  corresponded to the times of urine collection, and the values of the function  $V_I$  at these times were obtained by the method already described. Values of  $P_1$  and  $P_2$  were obtained from the plasma inulin-time curve. They were expressed in terms of mg inulin/100 ml plasma water. The constants of the line between the points  $t_1(V_I)_1$  and  $t_2(V_I)_2$  were calculated for each case†. Introducing the values for  $b$ ,  $P_1$  and  $P_2$ ,  $(V_I)_1$  and  $(V_I)_2$  into formula (8), a value for  $C$  was derived for each case.

In the use of the formula, it is convenient to employ values for  $(V_I)_1$  and  $(V_I)_2$  expressed in terms of hundreds of ml. By so doing, the values of  $C$  obtained can be directly converted to values which represent the plasma clearance per minute by the multiplication of the values for  $C$  by the factor

$$\frac{10,000}{100 - \text{g of plasma protein } 100 \text{ ml}}$$

For ordinary purposes, the plasma protein concentration may be taken as 6 g per 100 ml, so that the renal clearance of inulin equals  $C \times 106$ .

\* The use for  $P$  of a value  $2\frac{1}{2}$  minutes prior to the mid point of the clearance period, as the mean plasma level of inulin for the period is of course an approximation. It is, however, justified on the basis that the value obtained in this way does not differ significantly from the value obtained by estimating the area subtended by the plasma inulin time curve between the limits of the time  $2\frac{1}{2}$  minutes before the beginning and  $2\frac{1}{2}$  minutes before the end of the clearance period and dividing by the abscissa, provided the period is one occurring later than 30 minutes after injection.

† The analytical derivation of the constants of a line proceeds as follows —

Let the line  $V_I = a + bt$ . Take two points on the line  $t_1(V_I)_1$  and  $t_2(V_I)_2$ .

Then  $(V_I)_1 = a + bt_1$  and  $(V_I)_2 = a + bt_2$ . It follows that

$$a = \frac{t_2(V_I)_1 - t_1(V_I)_2}{t_2 - t_1} \quad \text{and} \quad b = \frac{(V_I)_2 - (V_I)_1}{t_2 - t_1}$$

*The Determination, by the Use of Formula (9), of the Error involved in the Assumption of Linearity of the Function  $V_I$  between the Times  $t_1$  and  $t_2$*

Equation (9) may be solved for  $P_0$  by substituting an experimentally determined value for  $P$ , i.e.  $P_1$  at time  $t_1$ , and using (1) the values of  $a$  and  $b$  calculated from the line  $V_I = a + bt$  between the points  $t_1(V_I)_1$  and  $t_2(V_I)_2$ , and (2) the value of  $C$  obtained by the use of formula (8). Employing this calculated value for  $P_0$ , values for  $P$  at varying values of  $t$  are obtained. The extent to which these derived values of  $P$  differ from those given by the graph constructed on the basis of experimentally determined values is a measure of the error involved in the use of formulae (8) and (9), i.e. in the assumption that the function  $V_I = \frac{I_b}{P}$  is linear between the time limits selected.

## RESULTS II

In no subject, following the injection of inulin, did the logarithm of the experimentally determined plasma concentration of inulin bear a linear relationship to time. A smooth curvilinear relationship existed (fig. 3), confirming the theoretical conclusion that, under the conditions of the experiment, the falling plasma curve is not exponentially related to time.

Table II presents all the experimentally determined clearances and the average for each subject. The average values range from 52 ml to 137 ml per minute, and therefore cover a wide range.

The results of the calculated plasma clearance of inulin, employing formula (8), are shown in Table III. The limits of time,  $t_1$  and  $t_2$ , between which the volumes  $V_I = \frac{I_b}{P}$  are assumed to be linear, are recorded, as are the values obtained for  $V_I$ ,  $(V_I)_1$  and  $(V_I)_2$  at those times, and the constants of the lines. The table includes also the plasma inulin levels,  $P_1$  and  $P_2$ , expressed as mg/100 ml plasma water and derived from the experimentally constructed inulin-time curve at  $t_1$  and  $t_2$ . In addition to the values for  $C$  obtained by the use of formula (8), the table presents the results of the conversion of  $C$  to actual plasma clearance values. For subjects 2 to 9 these are derived from data obtained in the same experiments as yielded the values given in Table II. The four calculated clearance values for subject No. 1 (J.R.) are derived from data obtained in four experiments carried out at approximately weekly intervals. The experimental clearance for this subject, shown in Table II, was obtained from a fifth experiment. For convenience in comparison, the experimentally determined average values for clearance (Table II, column 3) are shown, in brackets, in column 8 of Table III, after the corresponding calculated values.

There is excellent correspondence between the average inulin clearance values experimentally determined and the values calculated for the eight subjects in whom the two values were obtained from data accumulated from one experiment. The difference ranges from 0 to -3.5 ml/minute. In subject No 1 (J R), in whom five clearance

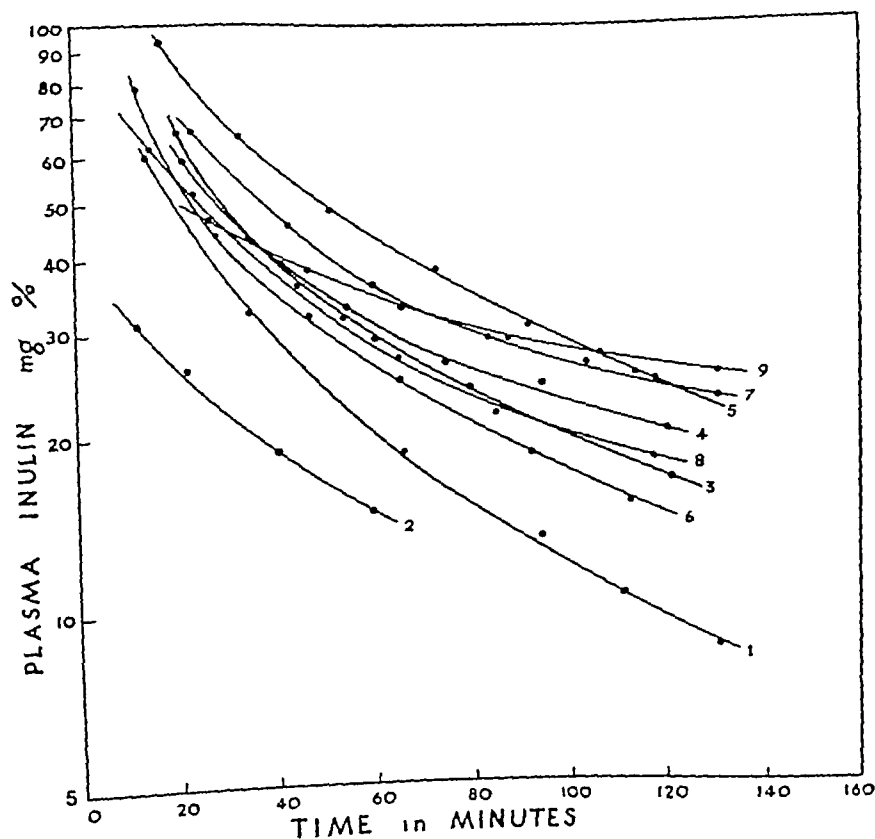


FIG 3—Plasma inulin concentration in mg/100 ml plotted semi logarithmically against time for the nine cases. In each subject the curve was drawn by visual approximation. Zero time corresponds to the mid point of inulin injection. The numbers indicate the subjects as listed in the tables.

determinations were performed on five separate occasions, the difference ranges from -6 to +3 ml/minute.

Results obtained by the use of formula (9) are shown in Table IV. Values of plasma-inulin concentration, expressed as mg/100 ml plasma water, obtained from the plasma inulin-time curve constructed from experimentally determined values, are compared with values obtained by the use of the formula. The differences,  $\Delta$ , between the results are given.

TABLE II — EXPERIMENTALLY DETERMINED CLEARANCE VALUES

1 Subject	2 Experimental clearance at each clearance period, ml /min	3 Experimental clearance Average ml /min	4 Average experimental clearance corrected to 1.73 sq m surface area, ml /min
1 J R	144.7 129.4	137	127
2 G S	113.9 94.0 90.0	99	101
3 W.H	100.7 97.2 92.8 82.4 86.3	92	101
4 M R	94.2 97.3 85.4 81.8	90	96
5 M F	84.9 83.3 78.2 76.9	81	80
6 M M	77.8 79.6	79	97
7 H L	71.0 78.7 75.6 75.1 67.0 73.8	74	71
8 A.A	76.3 74.6 61.4	71	67
9 J O	54.1 58.2 59.6 47.7 42.2	52	54

TABLE III — CALCULATION OF CLEARANCE  $C = \frac{b(\log P_1 - \log P_2)}{\log (V/I)_2 - \log (V/I)_1} - b$ 

1 Subject	2 Insulin given mg/kg body weight	3		4		5		6		7 $C$	8 Plasma clearance of insulin ml per minute $= C \times \frac{100 - g \text{ plasma protein}}{100 \text{ ml plasma}}$
		$t_1$ (mins)	$t_2$	$(V/I)_1$ in 100 ml	$(V/I)_2$	$a$	$b$	$P_1$ mg per 100 ml	$P_2$ plasma water		
1 J R	40.7	54	120	123	145	105.0	0.3333	12.2	5.4	1.317	140.1 (137)
	100.4	30	117	109	187	82.10	0.8966	33.8	10.3	1.306	138.9
	146.4	26	120	101	190	76.38	0.9468	66.5	15.0	1.283	130.5
	217.0	30	120	103	204	69.33	1.1222	92.6	22.1	1.231	130.0
2 G S	62.0	26	68	111	161	80.05	1.1905	25.8	13.4	0.9115	98.0 (90)
3 W H	126.5	40	120	95	122	81.50	0.3375	44.7	18.9	0.8231	88.5 (92)
4 M R	146.0	35	120	127	165	111.35	0.4471	44.7	21.2	0.8270	88.1 (90)
5 M F	137.5	32	117	93	142	74.55	0.5705	69.0	26.5	0.7445	80.1 (81)
6 M M	112.0	38	119	87	121	71.05	0.4108	30.4	15.6	0.7592	80.8 (76)
7 H L	130.7	37	118	121	184	92.22	0.7777	55.3	25.0	0.6953	73.9 (74)
8 A A	101.9	30	103	139	284	79.41	1.9863	50.5	10.0	0.6415	69.2 (71)
9 J O	98.1	40	121	102	131	87.68	0.3580	43.0	23.7	0.5142	55.2 (52)

1 or convenience in comparison, the average values for the experimentally determined clearances corresponding to the values obtained by use of the formula, are shown in brackets in column 8



At times later than  $t_1$  there is good correspondence between the values obtained from the curve and those obtained by calculation. At these times,  $t$  later than 26 to 40 minutes after injection, calculated values of  $P$  deviate from experimental values by amounts which vary from  $-1.5$  to  $+2.3$  mg/100 ml plasma water.

These deviations are of similar magnitude to the chemical error in the analysis of plasma inulin. In concentrations of 30 mg/100 ml plasma, the limits of chemical error were found to be  $\pm 1.26$  mg/100 ml plasma, ( $P=99$ ) and in concentrations of 50 mg/100 ml plasma, to be  $\pm 3.79$  mg. The maximum limits of chemical error are represented by the latter value, and their magnitude arises from the fact that, at concentrations of 50 mg/100 ml plasma and above, the deviation from the linear relationship between light transmittance and concentration becomes greater. Samples having an inulin concentration above 50 mg/100 ml plasma are suitably diluted, so that the error is again reduced.

Deviations of the calculated values of  $P$  from experimental results are greater when formula (9) is applied at time  $t=20$  minutes. They vary from  $-0.4$  to  $-15.2$  mg/100 ml plasma water. This results from the fact that at this time the curves relating  $V_I$  to time are far from linear, and the error in assuming a linear relationship is therefore considerable.

Evidently no significant error is introduced into formula (8) by the assumption that, under the conditions stated, the function  $V_I$  is linear with time.

## DISCUSSION

The work of Shannon and Smith [1935] and of Miller, Alving and Rubin [1940] appears to establish the existence of a direct proportionality between the rate of removal of inulin from the body and the plasma-inulin concentration between the limits of 5 and 400 mg per 100 ml. The claim that, as a necessary consequence of this relationship, the falling plasma-inulin concentration following a single injection of inulin into the blood-stream is exponentially related to time was made by Shannon and Smith [1935] and invoked by Alving and Miller [1940], Barnett [1940], and Newman, Bordley and Winternitz [1944]. This conclusion cannot be made from data which merely relate the plasma concentration of inulin to the rate of inulin loss from the body, for this implies that the rate of loss of inulin from the blood represents the rate of loss from the body. There is no evidence to suggest that this is so, nor is it self-evident.

On the assumption that inulin is excreted at a constant rate solely by glomerular filtration, an essential condition for the development of a falling plasma-concentration curve exponentially related to time is the diffusion of inulin, after injection, throughout its volume of

TABLE IV—COMPARISON OF VALUES FOR THE PERCENTAGE CONCENTRATION OF INULIN IN PLASMA WATER DERIVED FROM THE BEST CURVE DRAWN THROUGH EXPERIMENTALLY DETERMINED POINTS WITH THOSE CALCULATED ACCORDING TO THE FORMULA

$$P = P_0 \left( 1 + \frac{bt}{a} \right)^{-\frac{(c+b)}{b}}$$

Subject											
1 J R											
Inulin 19.7 mg/kg	t	20	30	54 (f)	70	90	105	120			
	P	25.5	17.9	12.2	9.6	7.7	6.5	5.4			
	P	19.7	17.0	12.2	9.9	7.7	6.5	5.4			
	A	- 5.8	- 0.0	0	+ 0.3	0	0	0			
Inulin 100.4 mg/kg	t	20	30 (f)	50	74	90	110	117	140		
	P	51.2	38.8	25.7	17.8	14.3	11.3	10.3	8.1		
	P	48.0	38.8	20.7	18.1	14.5	11.2	10.3	8.0		
	A	- 3.2	0	+ 1.0	+ 0.3	+ 0.2	- 0.1	0	- 0.1		
Inulin 146.4 mg/kg	t	20	26 (f)	60	90	120	150	180			
	P	87.2	66.5	33.6	21.0	15.0	11.8	9.6			
	P	76.2	66.5	34.6	21.8	15.0	10.8	8.1			
	A	- 11.0	0	+ 1.0	+ 0.8	0	- 1.0	- 1.5			
Inulin 217.0 mg/kg	t	20	30 (f)	58	90	120	147	165			
	P	133.0	92.6	51.1	31.0	22.1	16.5	10.5			
	P	117.8	92.6	53.0	32.3	22.1	16.5	10.5			
	A	- 15.2	0	+ 1.9	+ 0.4	0	0	0			
2 G S	t	20	26 (f)	48	68	100	120	120			
	P	27.1	25.8	17.7	13.4	10.0	8.1	6.5			
	P	26.7	25.8	17.7	13.4	10.0	7.7	6.5			
	A	- 0.4	0	0	0	0	0	0			
3 W H	t	20	40 (f)	50	81	100	120	120			
	P	71.0	44.7	31.4	20.3	16.0	12.0	10.0			
	P	57.7	44.7	30.0	28.1	23.1	18.9	16.0			
	A	- 13.3	0	+ 1.6	+ 1.8	+ 1.1	0	0			

4	M R	Experimental Calculated	<i>t</i>	20	35 ( <i>f</i> )	55	75	95	120
			<i>P</i>	61.7	44.7	34.9	28.5	25.2	21.2
			<i>P</i>	51.9	44.7	36.8	30.8	25.9	21.2
			$\Delta$	- 9.8	0	+ 1.9	+ 2.3	+ 0.7	0
5	M F	Experimental Calculated	<i>t</i>	20	32 ( <i>f</i> )	51	72	92	117
			<i>P</i>	93.0	69.9	52.2	40.9	32.9	26.5
			<i>P</i>	83.1	69.9	54.1	42.0	33.9	26.5
			$\Delta$	- 9.9	0	+ 1.9	+ 1.1	+ 1.0	0
6	M M	Experimental Calculated	<i>t</i>	20	38 ( <i>f</i> )	58	78	98	140
			<i>P</i>	59.5	39.4	28.9	22.9	18.7	15.6
			<i>P</i>	50.9	39.4	30.4	24.0	19.3	15.6
			$\Delta$	- 8.6	0	+ 1.5	+ 1.1	+ 0.6	0
7	H L	Experimental Calculated	<i>t</i>	20	37 ( <i>f</i> )	56	77	97	147
			<i>P</i>	74.5	55.3	43.1	34.6	29.3	25.0
			<i>P</i>	68.4	55.3	44.5	35.9	29.8	25.0
			$\Delta$	- 6.1	0	+ 1.4	+ 1.3	+ 0.5	0
8	A A	Experimental Calculated	<i>t</i>	20	30 ( <i>f</i> )	51	71	103	120
			<i>P</i>	64.5	50.5	36.6	28.0	19.6	16.1
			<i>P</i>	61.8	50.5	35.6	27.4	19.6	16.9
			$\Delta$	- 2.7	0	- 1.0	- 0.6	0	+ 0.8
9	J O	Experimental Calculated	<i>t</i>	20	40 ( <i>f</i> )	62	81	101	150
			<i>P</i>	60.6	43.6	35.5	30.6	26.7	23.7
			<i>P</i>	52.0	43.6	36.4	31.4	27.2	23.7
			$\Delta$	- 8.6	0	+ 0.9	+ 0.8	+ 0.5	0
			<i>t</i>	20	37 ( <i>f</i> )	56	77	97	176
			<i>P</i>	74.5	55.3	43.1	34.6	29.3	25.0
			<i>P</i>	68.4	55.3	44.5	35.9	29.8	25.0
			$\Delta$	- 6.1	0	+ 1.4	+ 1.3	+ 0.5	0
			<i>t</i>	20	40 ( <i>f</i> )	62	81	101	177
			<i>P</i>	60.6	43.6	35.5	30.6	26.7	23.7
			<i>P</i>	52.0	43.6	36.4	31.4	27.2	23.7
			$\Delta$	- 8.6	0	+ 0.9	+ 0.8	+ 0.5	0

distribution and the maintenance thereafter of a concentration in the plasma proportional to the mean concentration in the extravascular compartment of the volume of distribution. The data reported in this paper show that, between the limits of inulin dosage employed (49.7 mg to 217.0 mg per kg body-weight), equilibration, in this sense, does not occur. Therefore the falling plasma-inulin concentration is not exponentially related to time, and attempts to derive a value for renal clearance solely from the slope of the plasma-time curve when plotted semi-logarithmically cannot be successful, because the slope is not a simple function of the renal clearance.

The factors which determine the rate of the diffusion of a substance throughout the body after its injection into the blood-stream are not easily measured. They may include the rate of utilisation in the body, the rate of excretion, the diffusion constant of the substance employed, and the permeability of the capillary endothelium through which the diffusion occurs. Data which demonstrate the change in the volume of distribution with time do not elucidate the relative importance of the contributory factors, they demonstrate merely that, between defined limits of inulin dose and time, the ratio of the mean concentration of inulin in the extravascular compartment of its volume of distribution to the concentration in plasma water changes at a rate which is approximately constant. The assumption that this rate of change is in fact constant leads to a method for its determination through the evaluation of the slope of the line  $V_t = a + bt$ , with an error of no greater magnitude than that involved in the chemical determination of inulin in the plasma.

The approximation to the measurement of the rate of change of the function  $V_t$  permits the development of a formula for the renal clearance of inulin from the falling plasma inulin-time curve. The correspondence between the results obtained by the use of the formula and the averages of the results obtained by direct measurement (collecting urine by catheter over short periods of time) has been shown to be excellent.

In the application of the formula it is necessary to estimate values of the function  $V_t$  at only two times following the intravenous injection of an accurately measured amount of inulin (conveniently 100 to 120 mg/kg). In practice, it is convenient to choose times approximately 35 and 120 minutes after injection. Each value is calculated from a knowledge of the total quantity of inulin in the body, gained by subtracting from the amount injected the total amount which has been excreted. Since a knowledge only of the total amount is required, there is no necessity to empty the bladder prior to injection. On the contrary, it is advised that the subject have a full bladder at the time of injection, so increasing the accuracy of the first collection of urine 35 minutes later. The total urine excreted between 35 and 120 minutes is also required. The length of time between the collections is great

enough to allow spontaneous passage of urine without loss of accuracy, in at least the vast majority of subjects. It is not advisable to determine the value for  $V_1$  before 30 minutes after injection, the error introduced by so doing has been shown to become larger than the chemical error of the determination of inulin in the plasma.

In addition, two blood samples taken at the times of urine collection are required. Alternatively, the plasma-inulin concentrations at the times of urine collection may be derived from a curve constructed from inulin determinations made on several blood samples taken at other times and plotted against time. The values obtained for plasma-inulin concentration should be expressed in mg/100 ml plasma water before introduction into the formula.

The employment of the formula in human subjects allows the derivation of a value for the renal clearance of inulin which represents an average for the period over which it is applied. Apart from the calculative error, which has been shown to be of no greater magnitude than the chemical error of the estimation of inulin in the plasma, the accuracy of the formula is dependent upon the magnitude of the experimental errors introduced in the estimation of its constituents. It possesses obvious theoretical advantages over all methods which involve an approximation to the measurement of an instantaneous rate of inulin excretion by the collection of urine by catheter over periods of time sufficiently short to avoid wide fluctuations in the rate of excretion of inulin, and sufficiently long to permit the collection of appreciable quantities of urine. No such approximation is involved in the method using formula (8). In addition, the accuracy in the collection of urine excreted over a single period of approximately 85 minutes is much greater than that in procedures involving collection over periods of 15 to 20 minutes. The practical disadvantages of catheterisation and bladder washouts are obviated.

The simplicity of the method in comparison to those previously described is attained without loss of accuracy. The greater degree of precision in the measurement of the constituents of the formula than is possible in the direct estimation of the rate of renal excretion of inulin, renders values for clearance obtained by its use less susceptible to experimental error.

#### ADDENDUM

In view of the relevance of the results we have obtained with the use of inulin to attempts to derive a value for the rate of glomerular filtration employing mannitol, one experiment was performed on a normal subject (case No. 1, J.R.) in which 29.2 g of mannitol, dissolved in approximately 270 ml of sterile distilled water, were given by intravenous injection over a period of 8 minutes. Urine was collected at intervals following injection, and the amount of mannitol remaining in

the body was calculated by subtraction of the amount excreted from the total amount administered. The values of the function,  $\frac{M_b}{P}$ , where

$M_b$  equals the total mannitol in the body and  $P$  equals the plasma concentration of mannitol/100 ml of plasma water, were calculated at intervals following injection. For the purpose of the calculation it is assumed that no significant destruction of mannitol occurs in the body during the period of the experiment. The values of the function  $\frac{M_b}{P}$

derived at times  $t=20, 40, 71, 101$  and  $131$  minutes following injection were  $13.1, 14.2, 16.6, 16.8$  and  $17.1$  litres respectively. It is thus seen that mannitol differs from inulin in that it diffuses more rapidly throughout its volume of distribution, and that about 80 minutes after injection it becomes equilibrated between the plasma and the extravascular compartment of the volume of distribution. The results of this experiment are in agreement with the work of Elkinton [1947].

The studies of Smith, Finkelstein and Smith [1940] suggest that mannitol is excreted from the body solely by glomerular filtration. Under these circumstances, the falling plasma concentration of mannitol is necessarily exponentially related to time. 80 minutes after injection Formula (4) may then be written

$$\frac{V_m}{P} \frac{dP}{dt} = -C_m,$$

where  $V_m$  = the volume of distribution of mannitol

$P$  = the concentration of mannitol per 100 ml plasma water

$C_m$  = the renal clearance of mannitol from plasma water

This may be solved for  $C_m$

$$C_m = \frac{V_m (\log P_1 - \log P_2)}{t_2 - t_1} \times 2.3026$$

The logarithms employed are to the base 10, and 2.3026 is the modulus of the natural system of logarithms with reference to the common system.  $P_1$  and  $P_2$  represent the plasma concentrations of mannitol expressed in mg/100 ml plasma water, at the times  $t_1$  and  $t_2$  after injection. The application of this formula to the falling plasma curve between 71 and 131 minutes following injection, where  $V_m$  equals 16.8 litres and  $P_1$  and  $P_2$  equal 102.0 and 71.0 mg/100 ml plasma water respectively, gives a value for plasma clearance/minute of 107.9 ml.

Between 20 and 71 minutes following the injection of mannitol, the value of the function  $\frac{V_m}{P}$  is not constant. If it is assumed that this function bears a linear relationship to time, formula (8), derived for

inulin clearance, may be applied. The value of plasma clearance obtained for the period 20 to 71 minutes after injection by this method equals 105.0 ml/minute. Both values correspond well with the experimentally determined clearance value, which in this case averages 103.4 ml plasma/minute. All these values are approximately 30 ml lower than the inulin clearance of the same case. This is in general agreement with the work of Berger, Farber and Earle [1947]. The mechanism of the depression is not, however, evident.

## SUMMARY

1 The assumption underlying methods for determining the renal clearance of inulin from the slope of the falling plasma curve of inulin concentration, following a single injection, is investigated.

2 The concept that, following the injection of inulin into the blood-stream, equilibration occurs between the plasma and the extravascular component of the volume of distribution is shown to be untenable. The conclusion that the falling plasma-inulin concentration is exponentially related to time is consequently incorrect.

3 The errors introduced in the determination of the renal clearance of inulin from the slope of the falling plasma inulin-time curve by the assumption of equilibration are discussed.

4 A formula is devised for the determination of the renal clearance of inulin incorporating functions which are demanded by the failure of the establishment of equilibration.

5 The formula is applied to the determination of the renal clearance of inulin in nine subjects possessing widely different clearance values. These calculated values are shown to agree closely with average values experimentally determined.

6 The calculative error introduced into the formula by the simplifying assumption that the volume of distribution of inulin is linearly related to time between defined limits of inulin dose and time, has been shown to be of no greater magnitude than the chemical error in the determination of inulin in the plasma.

7 The formula can be applied without the need for accurate collection of urine over short clearance periods. It necessitates merely the passage of urine approximately 35 and 120 minutes after the injection of a known amount of inulin, and two blood samples taken at the time of the passage of urine.

8 The simplicity of the method in comparison to those previously described is attained without loss of accuracy. The greater degree of precision in the measurement of the constituents of the formula than is possible in the direct estimation of the rate of renal excretion of inulin, renders values for clearance obtained by its use less susceptible to experimental error.

9 The relevance of the results obtained with inulin to the determination of the rate of glomerular filtration employing mannitol are discussed briefly

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OBSERVATIONS ON DYE EXCRETION THROUGH SYNOVIAL  
MEMBRANE AFTER LUMBOSACRAL SYMPATHECTOMY  
AND CIRCULATORY OBSTRUCTION By KWOK-KEW  
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IN 1940-41 Engel described experiments with dogs, cats and rabbits from which he claimed that depriving the knee-joint of its sympathetic nerve-supply by unilateral lumbosacral sympathectomy lessened the outflow of a dye from the blood-vessels of the synovial membrane of that joint. The phenomenon occurred despite marked local vasodilatation as determined by thermo-electric measurements in the lower quadriceps muscles, and Engel explained this paradox by postulating a permeability factor under the influence of the sympathetic nervous system. He suggested that sympathetic activity increases and sympathectomy decreases capillary permeability, and he concluded that such a factor might compensate for vasomotor changes.

Later [1943-44] he stated that regional sympathectomy reduced or prevented the local increase in capillary permeability in traumatic shock, and he advocated blockage of the regional sympathetic chain with novocain as a therapeutic measure in that condition.

Since data about the control of capillary permeability are of fundamental importance for pathology, it seemed worth while to repeat the experiments. The opportunity was taken of applying the basic technique to the problem of ischæmia.

#### METHODS

Twenty-one adult rabbits, weighing from 1.9 to 3.7 kg, were used. Nembutal supplemented by ether was used for anaesthesia.

*Injection of Dye*—Following Engel, 10 c.c. of 1 per cent aqueous solution of acid fuchsin were injected into the marginal vein of the ear. This was repeated whenever the dye concentration of the perfusates became too dilute. Usually two such administrations were required in each experiment.

*Perfusion Apparatus*—Two identical 10 c.c. Luer syringes were mounted firmly on each side of a 20 c.c. syringe (fig. 1). A cross-bar

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was attached to the piston of the central syringe so that, when it descends, it pushes forward simultaneously the pistons of the two laterally placed syringes. The central syringe was filled with water through a rubber tubing attached to its nozzle, and the tubing was clamped with a screw clip. Lead bars were placed as weights on the cross-bar. By regulating the outflow from the central syringe with the screw clip, the descending cross-bar can be made to push forward the pistons of the two laterally placed syringes so as to empty them at an

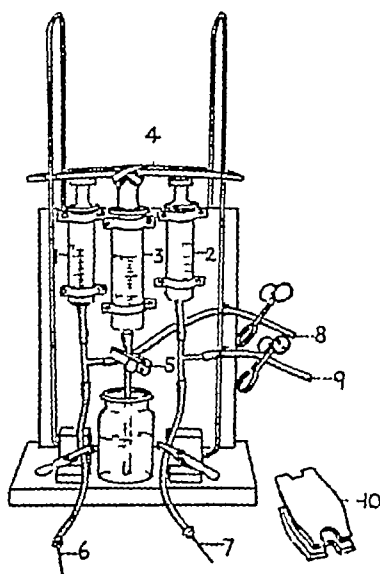


FIG 1—Perfusion apparatus. 1, 2, two 10 cc syringes for perfusion. 3, 20 cc syringe. 4, cross bar attached to the central 20 cc syringe. 5, screw clip regulating water outflow from the central syringe. 6, 7, perfusion needles connected by rubber tubing to the two perfusion syringes. 8, 9, side tubes for filling the perfusion syringes. 10, lead bars as weights on the cross bar.

identical steady rate. Each of the two 10 cc syringes was connected by rubber tubing to a needle 40 mm long and with 0.8 mm bore. Before use these syringes were filled with Ringer's solution.

*Perfusion Technique*—The needle connected to the tubing from the syringe containing Ringer's solution was inserted into each knee-joint medial to the patella. Some practice was needed to acquire confidence in the procedure. The clips were released, and after the joint cavity had been distended with 2–3 cc of fluid a similar needle, but with its head cut off, was inserted into the joint lateral to the patella to allow outflow of the perfusion fluid. The perfusates were collected from each knee-joint every 15 to 30 minutes, for a period of 2 hours or more. After adding two drops of acetic acid to regenerate

the dye, which becomes colourless in an alkaline medium, the dye concentrations of the perfusates from the normal and sympathectomised knee-joints were determined colorimetrically

The position of the needles was checked after each experiment. The knees were shaved several days before the perfusion. The two extremities were strapped down in identical positions during perfusion. Great care in inserting the needles was necessary in order to avoid bleeding. The perfusion fluid was not warmed to body temperature.

*Measurement of Quadriceps Temperature*—Two copper-constantan thermocouples were used for temperature estimations. The ends of each couple were hard soldered, inserted into a serum needle, and the junction was filed flat with the needle point. The control constant temperature junction of similar material was placed in a Dewar flask containing water at room temperature. Connections were made to a galvanometer through a switch and resistance box according to the method described by Lewis [1929-31]. Before each experiment the apparatus was calibrated to read the junction temperature changes directly from the scale. The scale could be read to a temperature change of 0.1 degree centigrade. The thermocouples were carefully compared before and after each experiment.

A thermocouple was inserted into each quadriceps muscle close to the patella. The temperature variations were recorded before and after the experiment and every 5 to 15 minutes throughout the experiment. The quadriceps temperature was assumed by Engel to represent that of the adjacent synovial membrane and to be proportionate to its blood-supply, other things being equal.

*Lumbosacral Sympathectomy*—With aseptic technique and under ether anaesthesia the anterior abdomen was opened through a median incision 10 cm long. Peritoneum covering the psoas muscle was incised and reflected medially. The inferior vena cava was retracted medially and the lumbosacral sympathetic chain was defined. After severing its rami communicantes the nerve was removed whole. The dissected nerves measured 6 to 14 cm and contained five to eight ganglia. The abdomen was finally closed in two layers with thread.

The control animals underwent sham operations.

After intervals of 2 to 28 days the knee-joints were perfused as described above.

*Vascular Obstruction*—After injection of dye the knee-joints were perfused, adjustment being made so as to give close conformity of the thermocouple reading and dye excretions between the two sides. A rubber tourniquet was then applied around a thigh and gradually tightened until variation in dye excretion was observed. In two rabbits the common and external iliac artery were ligated respectively and the quadriceps and calf muscle temperatures were recorded.

## RESULTS

*Effect of Lumbosacral Sympathectomy on Dye Excretion*

Ten rabbits were perfused 2 to 26 days after lumbosacral sympathectomy and five rabbits after sham operation. The results are summarised in Table 1, A. The muscle temperature on the sympathectomised side in seven animals showed no significant fluctuation from the

TABLE 1.—COMPARISON OF DYE EXCRETION AND QUADRICEPS TEMPERATURE  
A AFTER UNILATERAL LUMBOSACRAL SYMPATHECTOMY AND SHAM OPERATION  
B AFTER VASCULAR OBSTRUCTION BY TOURNIQUET

Rabbit No	Days after operation	Muscle temperature of affected side compared with normal side (average of 4 to 6 readings)	Ratio of dye excretion between affected and normal side (average of 4 to 6 readings)
<i>A Sympathectomised</i>			
1	2	+0.4° C	3.7 1
2	2	+2.5	3.5 1
3	3	+2.7	1.9 1
4	2	+3.0	1.7 1
5	2	+0.9	1.1 1
6	20	+0.5	1.2 1
7	22	-0.6	0.9 1
8	2	+0.9	1.0 1
9	9	+0.4	1.2 1
10	26	+0.9	1.0 1
<i>Control</i>			
1	2	0° C	0.9 1
2	2	+0.2	1.2 1
3	5	+0.6	1.0 1
4	20	-0.3	0.9 1
<i>B Vascular Obstruction</i>			
		(Average of readings)	(Average of readings)
1		-0.4° C	0.3 1
2		+0.1	0.6 1
3		-0.2	0.7 1
4		-0.3	0.2 1
5		-0.2	0.4 1

control values. In three it was raised. The dye excretion on the sympathectomised side in six cases was within the range of variation encountered in controls. It was, however, increased in four others, three of which were associated with increased muscle temperature. In no instance have I been able to substantiate Engel's observation of diminished dye excretion in the presence of increased muscle tempera-

ture on the sympathectomised side Fig 2, A and B, depict the result in a representative experimental and control animal respectively

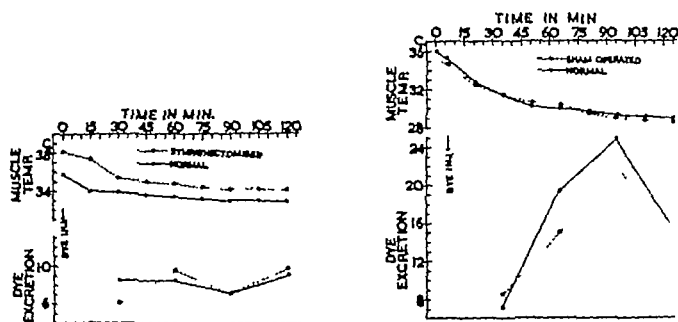


FIG 2—A. Relation of dye excretion and muscle temperature in rabbit 2 days after unilateral sympathectomy The dye excretion was expressed in terms of direct colorimetric reading of the perfusate dye concentration.

B Same in a control rabbit 2 days after sham operation Note similarity of the close association of dye excretion and muscle temperature between normal and operated sides in both sympathectomised and control animals

### *Effect of Vascular Obstruction on Dye Excretion*

Table 1, B, summarises the effect of tourniquet application on the quadriceps temperature and the dye excretion from the knee-joint Fig 3 illustrates a typical example The muscle temperature of the

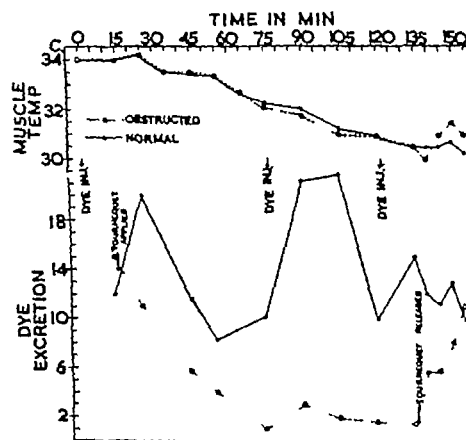


FIG 3—Relation of dye excretion and muscle temperature between normal and tourniquet-compressed limb Note marked fall in dye excretion on obstructed side whereas muscle temperature of the two sides did not show significant change

compressed side was little altered In contrast its perfusate dye concentration was reduced to a significantly low level It was noted, however, that the dye excretion was only modified after very high pressure

Unfortunately I could not record this pressure. On releasing the tourniquet after two hours' application a slight rise of muscle temperature above that of the normal side was observed. The dye concentration of the perfusates quickly returned to normal. Despite the increased quadriceps temperature there was not, up to forty-five minutes after removing the tourniquet, any significant increase in dye excretion on the affected side.

In the rabbits in which the common or external iliac artery was ligated no significant variation was noted in the temperature of the quadriceps or the calf muscle even after twenty-five minutes' obstruction, despite the fact that a profound vascular disturbance must have taken place with such a procedure.

### DISCUSSION

The status of the nervous control of capillary permeability is not clear [see Gellhorn's review, 1929]. Thus Bruck [1909] reported the production of unilateral oedema of the tongue by stimulating the glossopharyngeal nerve. Increased renal excretion of sodium chloride was observed after piqûre [Jungmann and Meyer, 1913] and sympathetic stimulation [Asher and Jost, 1914]. Unilateral cervical sympathetic ganglionectomy delayed the appearance of injected fluorescein in the anterior chamber of the eye [Kajikawa, 1922, Yamamoto, 1929] but hastened that of acid fuchsin and water blue [Karczag and Zilahy, 1925]. Kajikawa also showed that the protein content of the aqueous humour was diminished and the conjunctivitis induced by mustard oil was less intense on the operated side. However, Wesseley [1908] stated that after cervical sympathectomy both ocular pressure and protein content increased and more fluorescein appeared in the affected eye. According to Asher *et al* [1924] the chloride content of the saliva was lower on the sympathectomised side. Merz [1926] stated that the chloride content of the lachrymal secretion was decreased by sympathectomy. Hoffheinz [1931] observed after section of sciatic and femoral nerves that reabsorption from the knee-joint was considerably delayed.

Magnus-Alsleben and Hoffmann [1922] found that methylene blue stained paralysed muscle blue while normal muscles remained colourless. Yamamoto [1924] noted that indigocarmine injected into sympathectomised muscle appeared quicker in the urine than when injected into the normal muscle. An intracutaneous wheal also disappeared quicker from the sympathectomised side. He assumed that different anatomical regions have different capillary permeability. Alpern [1926] claimed greater penetration of injected neutral red, methylene blue and cyanol into the sympathectomised salivary gland than the normal organ. Urea, on the other hand, appeared later on that side than on the normal

side Gabbe [1926] found that colloidal dye appeared in the paralysed muscle in minute amounts only, whereas sugar, sodium chloride and urea injected intravascularly penetrated more quickly into the paralysed than normal muscles. However, these results were obtained only in frogs and not in mammals.

Linksz [1931] investigated the sympathetic and parasympathetic influence on the passage of fluorescein into the anterior chamber of the eye. He ascribed the variation in results obtained at different stages to the alteration of the sympathetic-parasympathetic equilibrium, and thought that sympathectomy increases the capillary permeability. Weiser and Rienmüller [1933] noted that vagotomy favoured intense staining of lung, muscle, etc. when trypan blue was injected into the larynx of frogs but sympathectomy gave negative results.

Most of the foregoing investigations suffer conspicuously from the failure of the investigators to take into account physical forces emphasised by Landis [1934] as playing an important part in the movement of fluid through the capillary wall. Of special importance is the concentration of the substance in the fluid, the hydrostatic pressure and membrane area. Evidently such factors will be pronouncedly affected by the rate of blood-flow. Engel measured the blood-flow by recording the quadriceps temperature, the assumption being that the temperature of a tissue is in direct proportion to its blood-flow. As already mentioned, Engel finally postulates from his experimental evidence the presence of a permeability factor under the influence of the sympathetic nervous system.

My investigations certainly do not confirm the claim of Engel. The discrepancy in the results is most likely due to technical errors. I have found that slight displacement of the thermocouple needle produces a noticeable temperature difference between the two sides. The relative position of the two perfusion needles in the joint cavity was also observed to affect profoundly the dye concentration of the perfusates. Thus a fortuitous dislocation of the thermocouple or the perfusion needle gave considerable errors. In my experiments much care was taken to obviate these faults. It may be mentioned that a close scrutiny of Engel's result reveals many inconsistencies.

Another point for criticism is Engel's assumption that the quadriceps temperature measures the state of blood-flow, *other things being equal*. Tissue temperature is an expression of the energy liberated by the tissue metabolism. The complicated process of tissue metabolism is, however, most unlikely to remain unaltered when the circulation is disturbed. Moreover, little divergence was observed in the muscle temperature when a tight tourniquet was applied to the limb. Even when the iliac artery was ligated to produce profound circulatory disturbance, the muscle temperature did not significantly change for twenty-five minutes. Thus the relation between the muscle

temperature and the blood-flow, if it exists, appeared under the existing experimental condition to be not a close one

In vascular obstruction the quadriceps temperature remained little altered in contrast to the significant reduction of the dye excretion in the joints. For the moment it is not possible to solve this problem with certainty without further work. It may be recalled that Landis [1928] reported that oxygen lack, if mild, had little effect, but prolonged and sustained anoxia increases the permeability of the capillary of frog's mesentery to fluid and to protein. In rabbit's ear Pochm [1939-42] noted that when its circulation was arrested and then released, a demonstrable oedema developed after 2 hours' occlusion and a massive oedema after 18 hours' occlusion. The oedema occurred as soon as the circulation was released. Calvin [1941] found that the plasma T 1824 and protein concentrations were significantly reduced only in terminal stages of fatal anoxia. Henry *et al* [1947] stated that there was no reason to anticipate changes in the capillary permeability in mild anoxia. However, in asphyxia, severe local ischaemia or other conditions in which the venous oxygen saturation may fall below a critical low level of 15 to 25 per cent, a significant increase in the permeability to protein occurred. Since anoxia affects the capillary permeability only significantly at a very low oxygen tension, a combination of factors such as ischaemia, anoxia, anaemia, etc is usually required if a sufficiently low level is to be reached. Mann *et al* [1938] studied the degree of vascular constriction necessary to affect the blood-flow through a vessel. The luminal area must be diminished 90 per cent before 50 per cent reduction in blood-flow takes place. Accordingly, it is doubtful if the extent of vascular obstruction caused by tourniquet application in the present experiment is adequate enough to produce anoxia of sufficient severity to affect the capillary permeability. Landis [1934] emphasised the capillary blood pressure as an important factor in determining the fluid movement through the capillary wall, and also showed that it is markedly raised in increased blood-flow occasioned by vasodilatation and active hyperaemia. Hence the decreased dye concentration after tourniquet application could be interpreted simply on the basis of lowered capillary filtration pressure consequent to diminished blood-flow. The question whether there is a coincidental increase in capillary permeability due to anoxia, but which is masked by the foregoing factor, is one which the present experiments cannot decide. This view is further supported by the observation that the dye excretion after releasing the tourniquet increased but slightly without significantly exceeding the control value.

#### SUMMARY

- 1 The claim made by Engel [1941] that lumbosacral sympathectomy gives a reduction in dye excretion into the knee-joint despite increased



muscle temperature and presumably increased blood-flow is not confirmed

2 Technical factors responsible for discrepant results are discussed Engel's assumption that muscle temperature measures local circulation, other things being equal, is criticised

3 Only severe vascular obstruction in the limb influences dye excretion in the knee-joint, probably because of decreased capillary pressure

#### ACKNOWLEDGMENTS

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ON HISTIDINURIA By R. KAPELLER-ADLER, Research Fellow  
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L-HISTIDINE normally occurs in the urine of pregnant women. Histidinuria can be demonstrated at a very early stage of pregnancy and persists throughout gestation [Voge, 1929, Kapeller-Adler, 1933, 1934, 1936, 1941*b*, 1943]. There is no histidine excretion in the urine of pregnant animals [Kapeller-Adler and Herrmann, 1934]. Results obtained in various investigations have revealed the existence of a relationship between the excretion of histidine and that of gonadotrophic hormones in human pregnancy, and have led to the theory that the increase of gonadotrophins in pregnancy may interfere with the normal enzymic breakdown of histidine by histidase [Kapeller-Adler and co-workers, 1935, 1936, 1937]. Page [1946] has put forward another explanation of pregnancy histidinuria, suggesting that this condition might be due to a decrease in the reabsorption of histidine in the renal tubules. This hypothesis is based on a study of the fate of L-histidine given intravenously or orally to pregnant and non-pregnant women.

The experiments now to be described were designed to test the validity of Page's hypothesis on a larger number of subjects. The effects of injected and ingested L-histidine on the blood and urinary histidine levels were simultaneously followed in 62 different women, of whom some were normally pregnant, some toxæmic and some not pregnant. The results do not support Page's theory, but confirm previous findings regarding a reduced enzymic breakdown of histidine in pregnancy.

#### METHODS

*Colorimetric Estimation of Histidine in Blood*—No chemical method specific for histidine is sensitive enough for the determination of this amino acid in the blood. In recent years Barac [1937] and Schwarz and co-workers [1938, 1939] have advocated the use of Pauly's diazo reaction for the determination of histidine in blood filtrates. In the present work a colorimetric estimation of histidine based on Pauly's diazo reaction and proposed by Macpherson [1946] was used with minor modifications.

*Procedure* Dilute 5 ml of citrated blood with 10 ml of distilled  $H_2O$  and add 10 ml 44 per cent (v/v) perchloric acid. Shake well and centrifuge. To 5 ml of the clear supernatant add 1 ml 1 per cent sulphamic acid in  $NHCl$  and 1 ml 5 per cent (w/v)  $NaNO_2$ . Mix and stand 5 minutes, shaking the test-tube from time to time. Blow in from a rapid delivery pipette 3 ml 20 per cent (w/v)  $Na_2CO_3$  (cryst.) and shake vigorously. Add 5 ml 20 per cent (v/v) ethanol and mix well. Read in the photoelectric colorimeter, using a green filter.

*Colorimetric Estimation of Histidine in the Urine*—The previously described method [Kapeller-Adler, 1941b] is slightly modified. A large excess of the bromine reagent is added to the urine and after 10 minutes removed by phenol. The tiresome testing with potassium iodide starch paper for an excess of bromine is thus eliminated, and the test becomes more accurate in inexperienced hands.

*Procedure* Dilute 5 ml of urine with 3 ml of distilled  $H_2O$  and add 2 ml of bromine reagent (5 ml of bromine and 500 ml of glacial acetic acid are diluted with 1000 ml of distilled  $H_2O$  and the solution stored in a brown bottle). Shake well and stand for 10 minutes. Add 0.2 ml of 10 per cent (v/v) phenol solution, shake well, and add 0.5 ml of a mixture of ammonia and ammonium carbonate (Mix 400 ml ammonia solution, sp. gr. 0.880, with 200 ml 10 per cent (w/v)  $(NH_4)_2CO_3$ ). Mix and place the tube into a beaker of boiling water for one minute. After cooling read in the photoelectric colorimeter, using a blue filter.

*Intravenous Injection*—In all cases the histidine content of the blood and of the night urine of the fasting patient was determined 24 hours before, immediately before, and 24 hours after the experiment. Twenty ml of sterile 10 per cent (w/v) L-histidine hydrochloride solution in saline, equivalent to 1.48 g free histidine, was rapidly injected intravenously, the patient was given one glass of lemonade every hour during the experiment. Blood specimens were withdrawn at regular intervals (5, 15, 30, 60, 90 and 120 minutes after the injection), and histidine estimations carried out on the citrated whole blood. Specimens of urine were carefully collected by means of an indwelling catheter after 30, 60, 90, 120 and 150 minutes. Volume and specific gravity were recorded and the histidine outputs determined.

*Oral Administration*—Histidine was again estimated in the blood and in the night urine 24 hours before and 24 hours after the experiment as described above. Four g of L-histidine hydrochloride, corresponding to 3 g of L-histidine, were given to the fasting women, and the patients were again advised to drink one glass of lemonade every hour during the experiment. Blood specimens were taken after 1, 2, 3 and 4 hours, and voided specimens of urine carefully collected at intervals of 4, 8, 12 and 16 hours after histidine ingestion.

## RESULTS

The results of all the experiments are presented in figs 1 to 8. Figs 1 to 4 represent results obtained after the intravenous administration, and figs 5 to 8 those found after ingestion of L-histidine hydrochloride. In each figure the blood histidine levels and the rate of histidine excretion (ordinate) are individually plotted against time (abscissa). On each curve is a number which identifies the patient, and also (in brackets) the number of weeks of gestation of the pregnant women.

*Intravenous Injection*—L-histidine intravenously injected to non-pregnant persons rapidly disappears from the blood-stream after an initial steep rise within the first 5 minutes after the injection (fig 1). No histidine was found in the urine except for small traces in a few of the specimens. Page [1946] obtained similar results when investigating 9 non-pregnant women. As long ago as 1908 Engeland found that after subcutaneous injections of histidine to dogs only minute amounts were excreted in the urine. This finding was confirmed by Abderhalden and co-workers [1910], Kotake and Komishi [1922], and by Kijokawa [1933]. Guinea-pigs after subcutaneous injections of L-histidine hydrochloride did not excrete histidine, or only a small proportion of it [Kapeller-Adler and Kohut, 1934, Eldbacher and Hertz, 1942].

When in this work histidine was injected into 8 normal pregnant women (fig 2) a completely different picture was obtained. As in non-pregnant persons, there was a steep rise in the blood histidine level within the first 5 minutes after the injection. But in contrast to the non-pregnant, all the pregnant women showed a very slow fall of the blood histidine within the first 2 hours after injection, although 24 hours after the injection it was found to have reached its initial value. Although the method used is not specific for histidine, it should be noted that the initial blood histidine values of pregnant persons were higher than those of non-pregnant ones. The blood histidine findings in this group of experiments are in contrast with the results of Page, who claims that injected histidine disappears at least as rapidly from the blood-stream of pregnant as from that of non-pregnant women.

All the pregnant women in this investigation showed a distinct histidinuria before the experiment. There was in all cases an increase persisting for a few hours, in some a very considerable one, in the rate of histidine excretion after histidine injection. The rate of histidine excretion had, however, returned to its initial value 24 hours after the injections. The stage of pregnancy appeared to be irrelevant, similar results having been obtained between the 20th and 39th week of gestation. It should be recorded here that pregnant guinea-pigs do not, on injection of histidine, excrete this amino acid in the urine [Kapeller-Adler and Kohut, 1934, Eldbacher and Hertz, 1942].

Women suffering from mild pre-eclamptic toxæmia were subjected

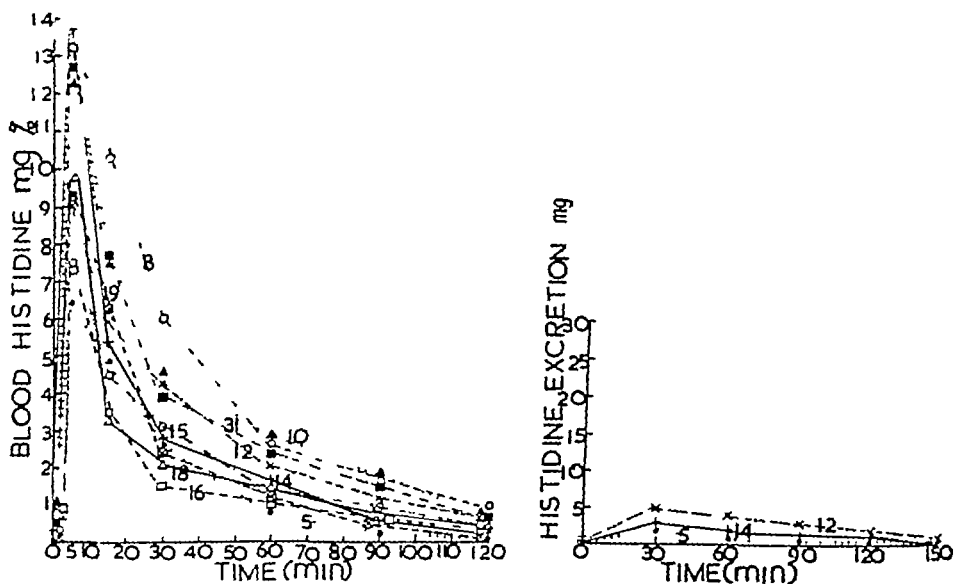


FIG 1—Non pregnant cases after 148 g L histidine i.v.

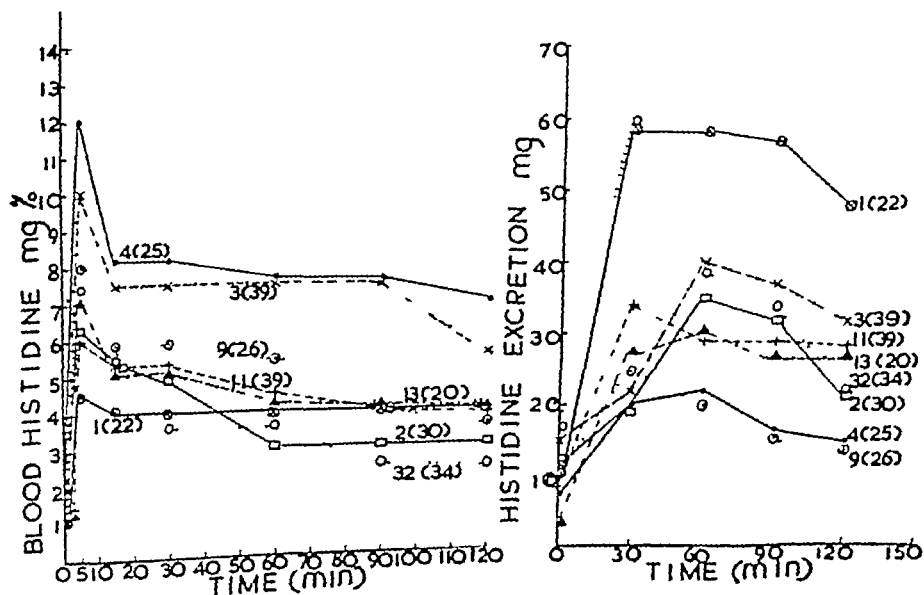


FIG 2—Cases of normal pregnancy after 148 g L histidine i.v.

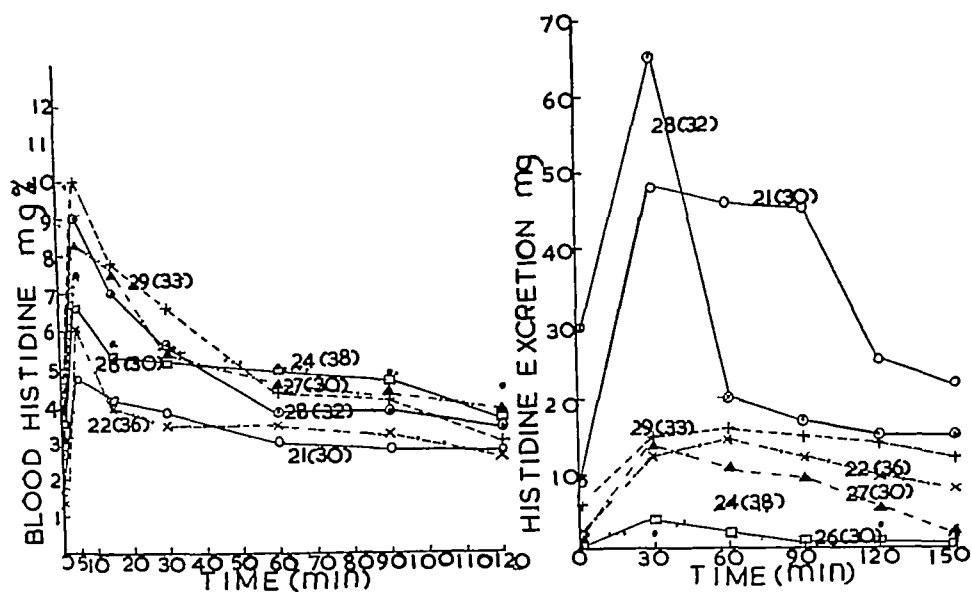


FIG 3—Cases of mild pre-eclamptic toxæmia after 1.48 g L histidine i.v

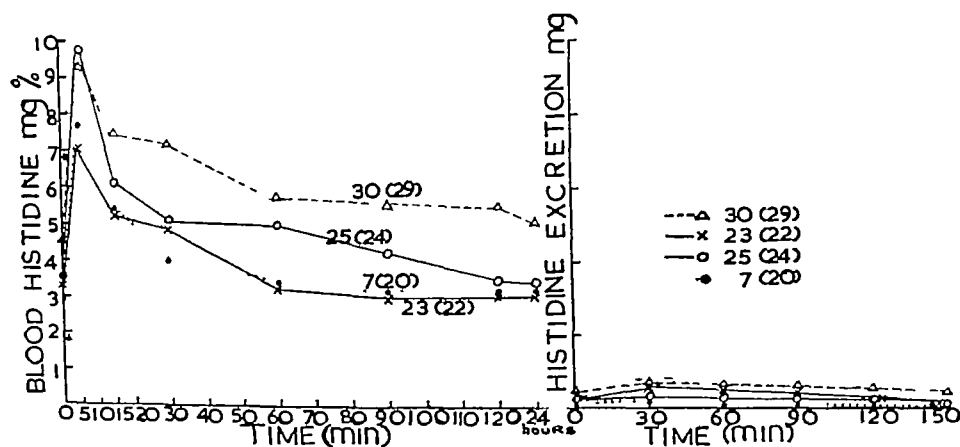


FIG 4—Cases of severe pre-eclamptic toxæmia after 1.48 g L histidine i.v

to the same treatment Fig 3 is representative of the results obtained Four out of 7 of the cases investigated showed a higher initial blood histidine level (2.5–3.8 mg per cent) than cases of normal pregnancy (about 2 mg per cent) As in normal pregnancy there was, on histidine injection, a very slow decline of the blood histidine, highly elevated within the first 5 minutes after the injection The initial value, in some cases rather high, was attained 24 hours after the injection

As to the histidine excretion, only 2 cases (Nos 21, 28) gave results comparable with those obtained in normal pregnancy In the remaining cases the slight initial histidinuria was followed by a small increase in the rate of excretion after the injection In 3 cases (Nos 24, 26, 27) the urinary histidine had already returned to the initial small value 150 minutes after the injection As can be seen from the corresponding blood histidine curve, the same 3 patients showed the highest blood histidine concentrations before and after the experiment The results obtained in this group of women seem to indicate that in some cases of mild pre-eclamptic toxæmia there is a tendency for the retention of histidine in the body This tendency appears to be even greater in women suffering from severe pre-eclamptic toxæmia (fig 4) All patients of this group showed a rather high initial blood histidine value (3–4.8 mg per cent) Histidine injection was followed by a steep rise of the blood histidine within the first 5 minutes, and after a slow decrease the normal high blood histidine level was reached 24 hours after the experiment The results found in the urine were striking In none of these cases was there more than a trace of histidinuria before, during, or after the experiment

These results clearly indicate that histidine both of endogenous and exogenous origin is not excreted, but seems to be retained in the bodies of women with severe pre-eclamptic toxæmia This observation confirms previous findings [Kapeller-Adler, 1941b, 1943], as a result of which it was concluded that a marked diminution, or total absence, of histidine excretion in pregnancy was a sign of toxæmia

*Oral Administration*—On oral administration of 4 g of L-histidine hydrochloride the blood histidine of non-pregnant persons rose to a peak within the first hour and fell rapidly within 3 to 4 hours after administration (fig 5) In only 2 out of the 10 cases studied a very small amount of histidine was found in the urine

Cases of normal pregnancy showed a smaller rise of the blood histidine within the first hour, followed by a comparatively slow fall (fig 6) At 4 hours after the oral dose of histidine the blood histidine was still high There was a considerable, in some cases even a very steep, rise in the rate of histidine excretion within the first 8 hours after the beginning of the experiment, irrespective again of the month of pregnancy

Twenty-four hours after histidine ingestion the blood and urinary



histidine values returned to the normal. Similar results in pregnant and non-pregnant persons are quoted by Page [1946] after oral administration of histidine. A marked increase in histidinuria on oral application of L-histidine hydrochloride to pregnant women was previously reported [Kapeller-Adler and Schiller, 1935, Neuweiler and Grimm, 1940].

On ingestion of histidine, the elevated blood histidine levels persisted in most of the women with mild toxæmic pregnancy longer than in normal pregnancy (fig 7). On the other hand, the urinary histidine output appeared to be in most of the cases smaller than under equal treatment in normal pregnancy. In all but 2 cases the rather low initial urinary histidine value was again attained after 24 hours.

Finally, on oral administration of histidine to women suffering from severe pre-eclamptic toxæmia (fig 8), the blood histidine content, highly increased in the first hour of the experiment, remained high during the first 4 hours after the ingestion, and in one case was still rather high even 24 hours after the experiment (No 19). In the urine of the same patient a very small amount of histidine was recovered 8 hours after the experiment had begun. In all the other specimens of urine of this patient, as well as in all the urine specimens of the other two women, only a trace of histidine was found.

## DISCUSSION

Page's observation that on intravenous injection histidine disappears from the blood-stream of pregnant women at least as rapidly as from that of non-pregnant women has not been confirmed in this work. On intravenous as well as on oral application histidine leaves the blood-stream of pregnant women very slowly, large amounts of histidine being excreted in the urine at the same time. In non-pregnant women the injected as well as ingested histidine disappears rapidly from the blood and hardly any trace of it is found in the urine. Such results, along with those obtained in this work in cases of toxæmic pregnancy, are incompatible with Page's view that a lowered renal threshold is the only abnormality of histidine metabolism in pregnancy. They support the previously made suggestion [Kapeller-Adler and Haas, 1935, Kapeller-Adler and Herrmann, 1936, Kapeller-Adler and Boxer, 1937] that the activity of histidase is inhibited in human pregnancy. Changes in the histidine metabolism occurring in pregnancy toxæmia as compared with normal pregnancy are best shown in the moderate and severe cases (figs 4 and 8), in which practically no histidine appeared in the urine in spite of the presence of high concentrations in the blood. This must have been due to renal failure. The milder toxæmic cases (figs 3 and 7) mostly gave results intermediate between those obtained in moderate and severe cases and the normally pregnant women. This retention of histidine in the body of women suffering from toxæmic

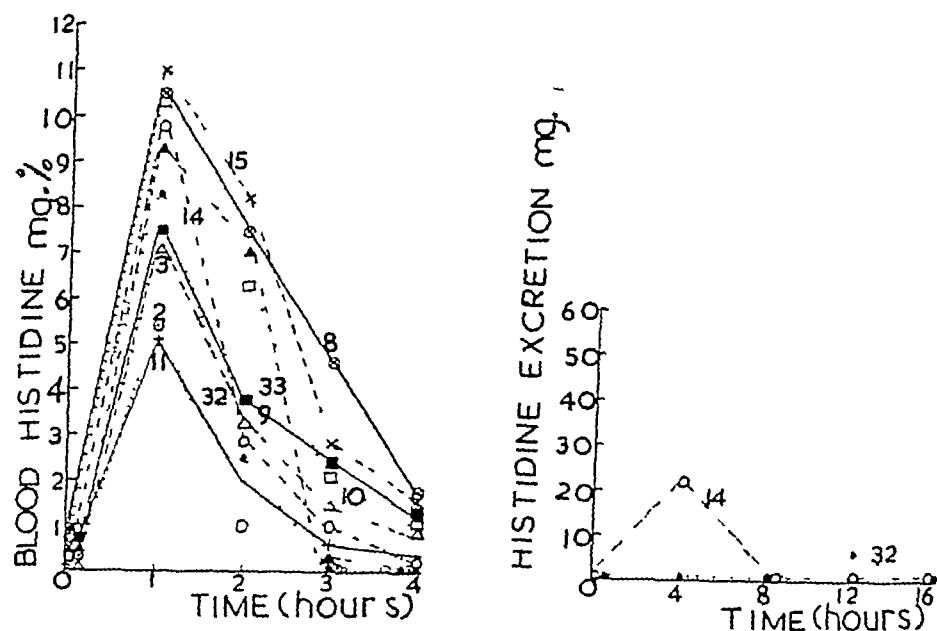


FIG. 5—Non pregnant cases after 3 g L-histidine per os

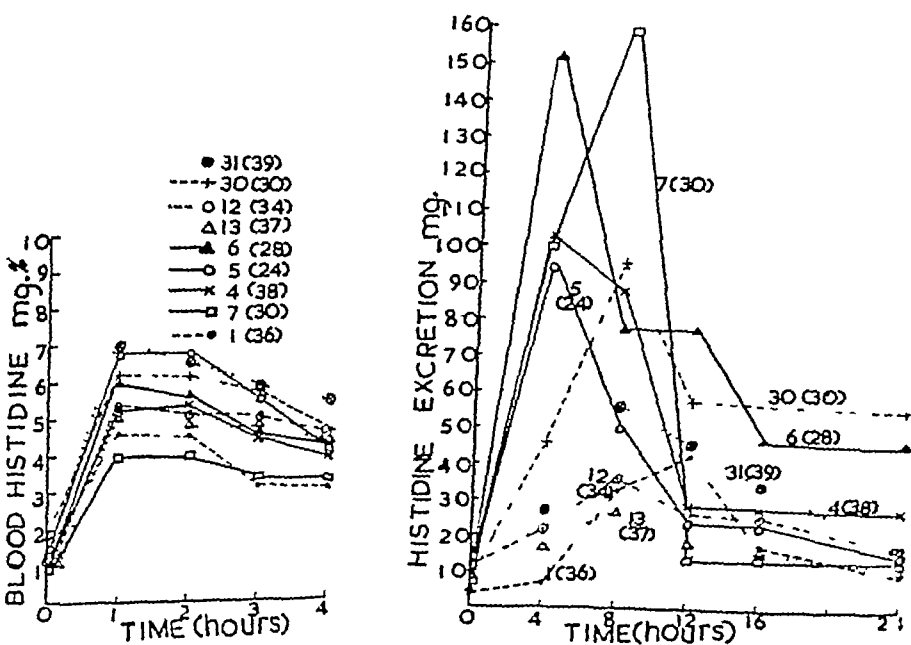


FIG. 6—Cases of normal pregnancy after 3 g L-histidine per os

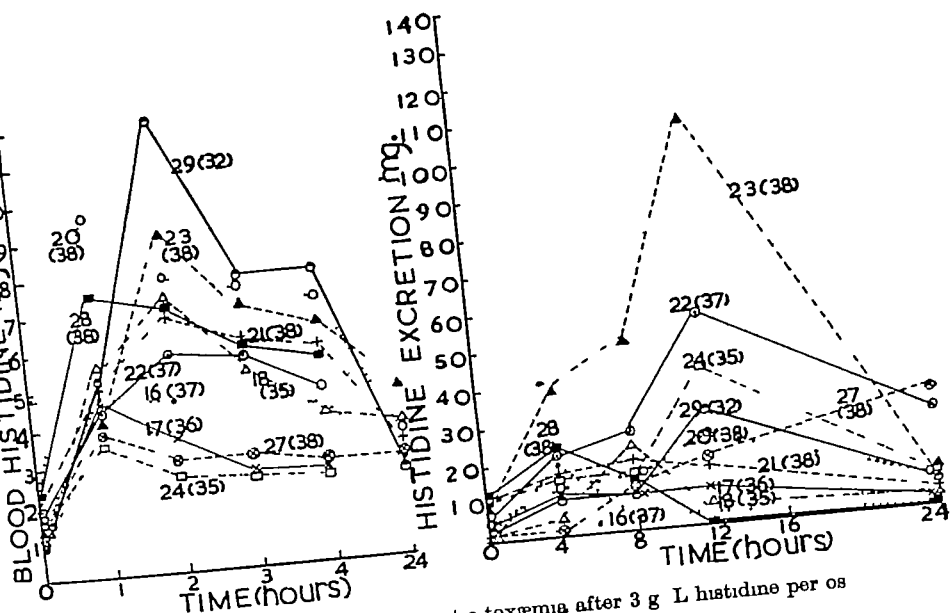


Fig 7—Cases of mild pre-eclampsia after 3 g L histidine per os

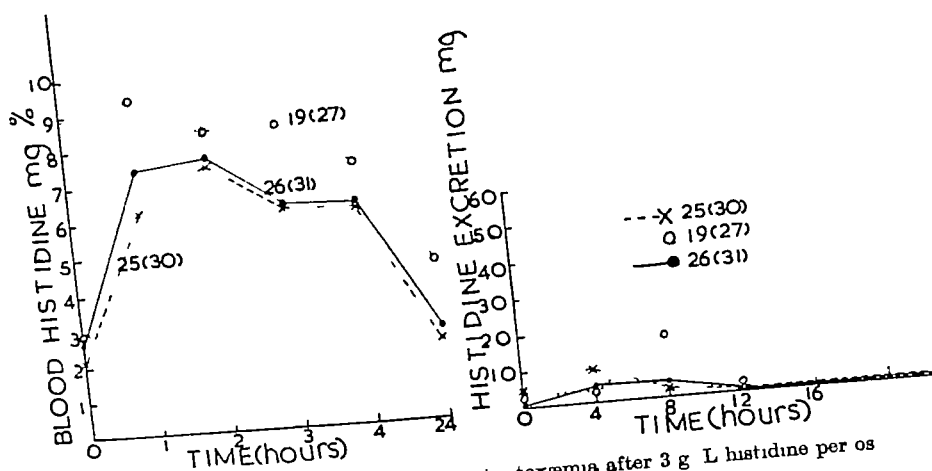


Fig 8—Cases of severe pre-eclampsia after 3 g L histidine per os

pregnancy gains importance with regard to the potential conversion of this neutral amino acid to its potent derivative, histamine [Kapeller-Adler, 1941a, 1941c, 1943]

Finally, Page has observed that in contrast with L-histidine, D-histidine on injection is largely excreted in the urine of both pregnant and non-pregnant women alike, high blood curves having been obtained in both groups. This is presumably due to the fact that, as Edlbacher [1926] has demonstrated, histidase metabolizes only L-histidine, leaving D-histidine intact.

### SUMMARY

1 L-histidine was given intravenously and orally to pregnant and non-pregnant women, and was estimated in the blood by a modified diazo reaction and by its reaction with bromine in the urine.

2 On intravenous as well as on oral application, L-histidine disappeared only very slowly from the blood-stream of pregnant women as compared with non-pregnant women, large amounts of it being simultaneously excreted in the urine. This observation does not confirm the results of Page, who found that on intravenous injection L-histidine leaves the blood-stream of pregnant women at least as quickly as that of non-pregnant persons. It seems, moreover, to support the previously suggested theory of a reduced activity of histidase in human pregnancy.

3 In severe toxæmia of pregnancy no histidine could be detected in the urine even after the injection or ingestion of histidine although the blood levels remained high. This indicates a retention of L-histidine by the kidney.

4 The results obtained in mild pregnancy toxæmia were intermediate between those found in severe toxæmia and in normal pregnancy.

### ACKNOWLEDGMENTS

I wish to thank Professor J. H. Gaddum, F.R.S., for valuable criticism and kind interest in this work. I am very much indebted to Professor R. J. Kellar for permission to use the clinical material. I am especially thankful to Professor Morris, Dr. Noble, and the nursing staff of the Department of Obstetrics of the Western General Hospital, Edinburgh, for valuable assistance in obtaining the clinical material.

My thanks are also due to the Medical Research Council for a whole time grant held when this work was carried out.

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THE PROTEIN-SPARING EFFECT OF CARBOHYDRATE IN  
NORMAL AND BURNED RATS By G H LATHE<sup>1</sup> and  
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With Statistical Note by R B FISHER

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It was shown by Cuthbertson, McGirr and Robertson [1939] that the excess nitrogen excretion during 9 days following the fracture of a femur could be reduced by providing the animals with additional food in the form of sucrose. It appeared that the local loss of nitrogen at the site of injury still persisted, though to a reduced extent, while the general loss was mitigated. These studies were not pursued further because, as they mentioned, the failing appetite of the injured animals limited the sucrose supplement consumed.

Since one of us [Lathe, 1949] has devised a technique of tube-feeding rats, in order to circumvent the difficulties of failing appetite after injury, it seemed of interest to extend the observations of Cuthbertson *et al* into the field of burns.

Previously we have been using for tube-feeding purposes a 25 per cent fat diet, which has the advantage of small volume. However, the addition of a sucrose supplement would change the character of the diet in two ways: it would increase the caloric intake, and it would change the carbohydrate-fat ratio. Since the latter is known to influence the nitrogen excretion, we have developed a non-fat diet to serve as a basal diet to which additional sucrose might be added.

## METHODS

The methods and the general plan of the experiments were the same as previously described [Lathe and Peters, 1949]. The 25 per cent fat diet was that previously used. The non-fat diet was prepared by the same procedure save that the arachis oil (42.2 ml) was replaced by an isocaloric amount of sucrose (91.8 g), water being added to maintain the previous volume. The method of tube feeding and of burning the rats was unaltered. The animals used were albino male rats of the same stock. All the experiments were conducted in a constant temperature

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room at 22° C. As previously, nitrogen excretion refers to urinary urea and ammonia nitrogen only. Faecal nitrogen was assumed to be constant, as shown by Croft and Peters [1945].

## RESULTS

### *Basal Nitrogen Excretion on 25 per cent Fat and Non-fat Diets*

In order to make a comparison possible with our previous experiments on tube-fed rats, it was necessary to ascertain whether the basal nitrogen excretion was different on a 25 per cent fat diet from that on the non-fat diet.

In Experiment 1, 15 rats were maintained on a 25 per cent fat diet for 5 days. They received 17 ml per day, or 16.8 cal/100 g of body-weight. Nitrogen excretion was measured during this period. Seven animals were then given the non-fat diet during a 5-day period, while the remainder continued on the 25 per cent fat diet. The nitrogen excretion and weight changes are summarized in Table I (full data being given in Appendices 1 and 2). The substitution of carbohydrate thus results in reduced nitrogen excretion.

TABLE I—EXPERIMENT 1. EFFECT UPON THE NITROGEN EXCRETION IN RATS OF SUBSTITUTING A NON-FAT DIET FOR DIET CONTAINING 25 PER CENT FAT

	Number of animals	Five day means of urea and ammonia nitrogen excretion (mg)			Weight change Days 6-11 (g)
		Preliminary period Days 1-5	Experimental period Days 6-10	Difference	
Group 1 Control	8	202	188	-14 ± 9.5 *	+4
Group 2 Diet changed to carbohydrate on day 6	7	205	173	-32 ± 5.13 *	+2

\* Standard error of the difference calculated from individual figures (Preliminary period on 25 per cent fat diet; experimental period 25 per cent fat diet for Group 1, non-fat diet for Group 2).

### *Effect of Sucrose Supplement between Meals*

The effect of burning and of a sucrose supplement was examined in Experiment 2. The animals from Experiment 1 were used. Each of the previous groups was divided into two groups. All animals were then burned, and each was given a supplement of either water or sucrose. The supplements were not given with the meals at 9 a.m.



and 5 p.m., but at 11 p.m. and 4 a.m. Thus the animals received two basal meals of either 25 per cent fat or non-fat composition, followed by two supplements of either water or sucrose solution. The sucrose supplements consisted of 4 ml of 30 per cent sucrose on the day after burning, followed by 50 per cent sucrose until the sixth day after burning, when the experiment was concluded. This represents a 62 per cent increase in calories above the basal diet, which provided 16.8 calories per 100 g for a 282 g rat.

It would have been desirable to have unburned control animals on supplementary feedings. A shortage of rats made this impossible. However, 4 albino rats of a different stock (Department of Pathology, Oxford) were obtained. These animals formed a fifth group. They were kept on a non-fat diet for 2 days, and then received the sucrose supplement without being burned. Nitrogen excretion and animal weights are given in Appendices 3 and 4 respectively. The effect of the supplement and of burning on the nitrogen excretion of the comparable groups, 1 to 4, are summarized in Table II. It will be noticed that the excess nitrogen excretion due to burning is similar on a 25 per cent fat diet and a non-fat diet. The addition of sucrose led to diminished nitrogen excretion from burning in the group fed 25 per cent fat. The difference between the excess nitrogen due to the burn on the 25 per cent fat diet, and on the 25 per cent fat diet supplemented with sucrose, is significant ( $P=0.05$ ).

TABLE II—EXPERIMENT 2 EFFECT OF BURNING ON MEAN DAILY UREA AND AMMONIA NITROGEN EXCRETION, ON FAT AND NON FAT DIETS, WITH AND WITHOUT SUPPLEMENTS

Group	Number of animals	Diet	Supplement	Mean daily nitrogen excretion (mg)		Change
				Basal period	Burn period	
1	4	Non fat	Water	167	209	+ 42 ± 3.6
2	3	Non fat	Sucrose	167	202	+ 35 ± 19.1
3	4	25 per cent fat	Water	192	227	+ 35 ± 8.1
4	4	25 per cent fat	Sucrose	183	190	+ 7 ± 19.7
5	4	Non fat	Sucrose	154	Not burned 163	+ 9 ± 3.9

#### *Effect of Sucrose Supplement with Meals*

At the time this experiment was conducted, account was taken of the effect of fat in retarding digestion of proteins by delaying gastric secretion [Babkin, 1944]. The possibility was considered that the sucrose supplement exerted its influence only on the 25 per cent fat

diet, because only on the fat diet was the protein in process of assimilation at the time the excess carbohydrate was being absorbed. The protein-sparing effect of carbohydrate has been shown to depend on the simultaneous administration of carbohydrate and protein. This has been demonstrated under various conditions by Larson and Chaikoff [1937], Cuthbertson and Munro [1937, 1939], Lovell and Rabinowitch [1939], and by Cuthbertson, Webster and Young [1940]. In order to test this possibility the sucrose supplements were fed with the protein-containing meals in Experiments 3 and 4.

In Experiment 3 all of the animals were on a non-fat basal diet for the 4 preliminary days. They were then divided into 5 groups. Groups 1, 2 and 3 were burned and received no supplement, a water supplement, and a sucrose supplement respectively. Groups 4 and 5 were not burned, the first receiving a water supplement, the second a sucrose supplement.

In order to give the extra sucrose with the meals, the basal diet was given as 4 meals, one every 6 hours, per day. The volume was 4.5 ml, and the supplements, of equal volume, were given immediately after the food. The sucrose supplement was a 50 per cent solution, representing a 70 per cent increase in calories during the 6 days after burning. The basal diet provided 17.9 calories per 100 g for a 280 g rat. It will be noted that the number of burned animals on a sucrose supplement was two. Initially there were 5 animals, but the increased concentration of sucrose in this experiment (particularly when given immediately after burning) proved to be too much, and 3 animals were lost. The nitrogen excretion and weights of the different groups are given in Appendices 5 and 6 respectively. The effects of burning on a non-fat diet and a supplemented non-fat diet are summarized in Table III. In this experiment there was an apparent reduction in the nitrogen excretion due to the sucrose supplement, the significance of the

TABLE III — EXPERIMENT 3 EFFECT OF SUCROSE GIVEN WITH MEALS AND EVERY 6 HOURS UPON NITROGEN EXCRETION AFTER BURNING

Group	Number of animals	Treatment	Supplement	Daily nitrogen excretion (mg)		Change
				Days 1-4	Days 5-10	
1	5	Burn	Nil	166	224	+ 58
2	4	Burn	Water	177	223	+ 46
3	2	Burn	Sucrose	191	185	- 6
4	5	No burn	Water	174	185	+ 11
5	4	No burn	Sucrose	168	148	- 20

Excess nitrogen excretion due to burn  
(mg per day)

On basal diet (46 - 11) = 35

Plus sucrose supplement (20 - 6) = 14

difference was made doubtful by the few animals left alive in the sucrose group

Experiment 4 was a repetition with minor changes of Experiment 3, made necessary by the mortality as mentioned above. There were 4 groups of animals. The preliminary period on a non-fat diet, of 5 ml every 6 hours (20.7 cal/100 g of body-weight for a 270 g rat), was 4 days. Two groups were then burned, and one of them and one control group were given sucrose supplements with their meals. The sucrose supplement was a 30 per cent solution on the day of burning and a 50 per cent solution for the remaining 5 days of the experiment. This represented a 62 per cent increase in the calorie intake. The nitrogen excretion and animal weights are given in Appendices 7 and 8. It will be noted that 2 of the burned animals without a supplement died at the end of the third day after burning. Of the 2 remaining animals in this group, 1 was killed at the beginning of the tenth day by a feeding error, the catheter having been introduced into the trachea. In this case the average nitrogen excretion for days 8 and 9 is entered in the table, instead of the average for a three-day period. All of the burned animals on a sucrose supplement survived. The effect of the burn is summarized in Table IV, and shows a slight reduction in the mean excretion due to carbohydrate feeding.

TABLE IV—EXPERIMENT 4 EFFECT OF SUCROSE GIVEN WITH MEALS AND EVERY 6 HOURS UPON NITROGEN EXCRETION AFTER BURNING

Group	Number of animals	Treatment	Supplement	Daily nitrogen excretion (mg)		Change
				Days 3-4	Days 5-10	
1	4	No burn	Nil	242	236	- 6
2	4 *	Burn	Nil	232	256	+ 24
3	4	No burn	Sucrose	223	193	- 30
4	5	Burn	Sucrose	224	207	- 17

Excess nitrogen excretion due to burn  
(mg per day)

On basal diet (24 + 6) = + 30

Plus sucrose supplement (30 - 17) = + 13

\* Original number of animals. In last period (see Appendix) 2 animals died. Allowance was made for this in the averages.

We are indebted to Dr R. B. Fisher (of this Department) for a statistical analysis of the results; he has contributed the following note.

#### A STATISTICAL ANALYSIS OF EXPERIMENT 4 By R. B. FISHER

The first point which is clear from the data of Appendix 7 is that there is a greater nitrogen excretion on days 3-4 than on days 1-2 in all

groups. Thus the mean nitrogen excretion on days 3-4 is likely to be a better estimate of what the nitrogen excretion might be expected to be in subsequent periods in the absence of experimental interference, than is the average taken over days 1-4. The data for days 1-2 have therefore been excluded from the analysis.

Secondly, since only two animals in Group 2 survived after the seventh day, and since the effect of burning on nitrogen excretion is normally manifest during the first three days after burning, it seems reasonable to confine the analysis to a comparison of the nitrogen excretions of days 3-4 with those for days 5-7.

The experimental treatments instituted on day 5 can be symbolized as

NN = no sucrose no burning

NB = no sucrose burning

SN = sucrose no burning

SB = sucrose burning

The best way of examining the effects of these treatments is to analyse the alterations in mean daily nitrogen excretion produced in each rat. In this way the effects of variation in the characteristic daily nitrogen excretions from rat to rat are largely eliminated.

The data, arranged for this purpose, are tabulated below.

	No burning		Effect (NN)	Burning		Effect (NB)
	Days	Days		Days	Days	
	3-4	5-7		3-4	5-7	
No sucrose	224	209	- 15	249	209	+ 20
	298	304	+ 6	221	252	+ 31
	233	237	+ 4	258	274	+ 16
	213	226	+ 13	244	258	+ 14
Mean effect			+ 2.00			+ 20.25

	No burning		Effect (SN)	Burning		Effect (SB)
	Days	Days		Days	Days	
	3-4	5-7		3-4	5-7	
Sucrose	234	215	- 19	224	175	- 49
	208	157	- 51	198	188	- 10
	215	167	- 48	241	207	- 34
	234	184	- 50	230	187	- 43
				225	183	- 42
Mean effect			- 42.00			- 35.60

The standard errors of the mean effects range from  $\pm 3.8$  to  $\pm 7.7$ , and all the means are significantly different from zero except NN, with a standard error of  $\pm 6.0$ . That is,

- (1) there is no evidence of further appreciable rise of nitrogen excretion on days 5-7, in the absence of experimental interference,
- (2) in the absence of sucrose there is a significant increase in nitrogen excretion in the first three days after burning,
- (3) the sucrose supplement significantly diminishes nitrogen excretion whether or no the animal is burnt at the same time

The effect of burning in the presence of a sucrose supplement can only be estimated by means of the difference between SB and SN, and is therefore  $(-35.60) - (-42.00) = +6.40$ . This is certainly much smaller than the NB effect, but it is unfortunately not directly comparable with it. The individual effects NN, NB, SN and SB are comparisons within individual groups of rats, but the difference (SB-SN) involves comparison between different groups of rats, and to obtain a fair comparison we must compare it with the corresponding difference (NB-NN). If we call these differences in the presence and absence of sucrose respectively  $B_s$  and  $B_n$ , we have

$$\begin{aligned} B_n &= (NB-NN) = +18.25 \pm 7.09 & P < 0.025 \\ B_s &= (SB-SN) = +6.40 \pm 10.28 \\ (B_n - B_s) &= +11.85 \pm 12.81 & P > 0.15 \end{aligned}$$

P here is the probability of obtaining a zero or negative mean difference in a repetition of the experiment. Although  $B_s$  is not significantly different from zero, it is obvious from its standard error that it is also not significantly different from  $B_n$ , so that the experiment unfortunately provides no basis on which to decide whether a sucrose supplement does or does not inhibit the increase in nitrogen excretion normally produced by burning.

*Analysis of Experiment 3*—The data of Experiment 3 have been analysed on similar lines. There is no significant difference between Groups 1 and 2 in this experiment, so that it has been taken that the water supplement did not significantly alter the nitrogen response to burning. Differences  $B_w$  and  $B_s$ , corresponding to those described above, were then computed for the water-and-sucrose-supplemented groups respectively. The results are

$$\begin{aligned} B_w &= +38.50 \pm 15.81 & P < 0.025 \\ B_s &= +17.00 \pm 13.70 \\ (B_w - B_s) &= +21.50 \pm 23.09 & P > 0.15 \end{aligned}$$

Here again the effect of sucrose on the response to burning is equivocal.

## DISCUSSION

The primary question with which this work is concerned is, "Does the provision of extra carbohydrate reduce the nitrogen excretion due to burning?" In the light of the statistical note, the answer obtained from Experiments 3 and 4 is that the data do not permit of a decision either way. Hence the answer reached is not more definite than previous ones in spite of the improvement in technique introduced by the tube feeding. It is hard to see how the technique can be further improved so far as the daily ingestion of food is concerned, yet even with similar daily doses of the diet the excretion of the burned rats showed such wide variations upon the high sucrose diet that the results were not statistically significant. To make them so would require such large numbers of animals that the technical difficulties seem insuperable. It is interesting to note the large reduction in nitrogen excretion induced by the sucrose feeding, and this may be held to be an indication that the provision of extra calories in this form is advisable in patients to prevent protein wastage.

## SUMMARY

1 Studies of the nitrogen excretion of tube-fed rats have been made under different dietary conditions, and following a standard burn.

2 Substitution of carbohydrate for the fat of a 25 per cent fat diet, without changing the caloric intake, results in a reduced nitrogen excretion.

3 In unburned animals a sucrose supplement given with the meals substantially reduces the nitrogen excretion on a non-fat diet during a 3-day period, following which it rises toward its previous level.

4 The data obtained upon the effect of sucrose supplements after burning, when examined statistically, did not permit of a decision either way.

5 The significance of these findings is discussed.

## ACKNOWLEDGMENTS

Our thanks are due to Miss Christine Stayte for technical assistance, to the Medical Research Council and Nuffield Committee (Oxford) for grants in aid of the research, and also to the Carnegie Grant Trustees.

## APPENDIX 1

## EXPERIMENT 1—AVERAGE DAILY UREA AND AMMONIA NITROGEN EXCRETION (mg)

Group 1	Days 1-3	Days 4-5	Days 6-7	Days 8-10
	25 per cent fat diet		25 per cent fat diet	
	208	240	203	173
	186	161	206	197
	218	200	219	171
	176	208	197	189
	229	211	189	167
	192	192	171	166
	218	208	171	216
	206	176	203	185
Mean	204	199	195	183

Group 2	25 per cent fat diet		Non fat diet	
	219	214	181	181
	211	224	165	161
	208	204	152	151
	212	219	173	174
	208	232	189	173
	183	172	152	155
	184	188	162	173
Mean	203	208	182	167

## APPENDIX 2

## EXPERIMENT 1—ANIMAL WEIGHTS (g)

Group 1	Day 1	Day 6	Day 11	Weight change (days 6-11)
	25 per cent fat diet	25 per cent fat diet	fat diet	
	289	278	281	
	284	279	281	
	274	270	274	
	267	262	268	
	286	283	288	
	284	300	307	
	273	281	285	
	264	269	270	
Mean	278	278	282	+ 4

Group 2	25 per cent fat diet	Non fat diet		
	320	306	308	
	284	274	279	
	275	280	282	
	272	273	277	
	312	297	299	
	289	299	302	
	270	285	288	
Mean	289	288	291	+ 2

## APPENDIX 3

EXPERIMENT 2 — AVERAGE DAILY UREA AND AMMONIA NITROGEN  
EXCRETION (mg)

	Days 1-2	Days 3-5	Days 6-11
Group 1	Non fat diet		Burned + water supplement
	181	181	216
	165	161	208
	152	151	202
	173	174	211
Mean	168	167	209
Group 2	Non fat diet		Burned + sucrose supplement
	189	173	200
	152	155	226
	162	173	181
Mean	168	167	202
Group 3	25 per cent fat diet		Burned + water supplement
	203	173	246
	206	197	234
	219	171	216
	197	189	214
Mean	206	182	227
Group 4	25 per cent fat diet		Burned + sucrose supplement
	189	167	188
	171	166	201
	171	216	181
	203	185	190
Mean	183	183	190
Group 5	Non fat diet Days 4-5		Not burned + sucrose supplement
	156		154
	138		157
	134		157
	188		183
Mean	154		163



## APPENDIX 4

## EXPERIMENT 2—ANIMAL WEIGHTS (g)

Weight change  
(days 6-12)

	Day 1	Day 6	Day 12	
Group 1	Non fat diet	Burned + water supplement		
	306	308	297	
	274	279	276	
	280	282	282	
	273	277	278	
Mean	283	286	283	- 3
Group 2	Non fat diet	Burned + sucrose supplement		
	297	299	308	
	299	302	318	
	285	288	302	
Mean	294	296	309	+ 13
Group 3	25 per cent fat diet	Burned + water supplement		
	278	281	282	
	279	281	274	
	270	274	276	
	282	288	273	
Mean	272	276	276	0
Group 4	25 per cent fat diet	Burned + sucrose supplement		
	283	288	304	
	300	307	320	
	281	285	301	
	269	270	293	
Mean	283	287	304	+ 17
Group 5	Non fat diet Day 4	Not burned + sucrose supplement		
	246	255	262	
	260	266	278	
	259	263	277	
	265	270	282	
Mean	257	263	275	+ 12

## APPENDIX 5

EXPERIMENT 3—AVERAGE DAILY UREA AND AMMONIA NITROGEN  
EXCRETION (mg)

	Days 1-4	Days 5-7	Days 8-10
Group 1		Burned + no supplement	
	180	246	247
	177	211	187
	150	241	198
	161	231	176
	163	259	249
Mean	166	238	211
Group 2		Burned + water supplement	
	184	210	227
	181	249	231
	178	234	193
	167	230	206
Mean	177	233	214
Group 3		Burned + sucrose supplement	
	210	186	214
	167	166	176
Mean	191	176	195
Group 4		No burn + water supplement	
	149	139	128
	199	189	198
	187	212	192
	166	204	177
	168	210	195
Mean	174	191	179
Group 5		No burn + sucrose supplement	
	158	136	167
	174	125	155
	168	129	161
	172	152	161
Mean	168	135	161

## APPENDIX 6

## EXPERIMENT 3 —ANIMAL WEIGHTS (g)

	Day 1	Day 5	Day 11	Change (days 5-11)
Group 1		Burned + no supplement		
	304	295	288	
	289	278	274	
	275	280	282	
	278	277	280	
	267	270	267	
Mean	283	280	278	- 2
Group 2		Burned + water supplement		
	300	301	308	
	289	285	275	
	274	275	277	
	269	271	267	
Mean	283	283	282	- 1
Group 3		Burned + sucrose supplement		
	299	287	303	
	268	271	297	
Mean	283	279	300	+ 21
Group 4		No burn + water supplement		
	291	294	301	
	283	299	279	
	273	269	273	
	268	269	276	
	260	253	255	
Mean	273	277	277	0
Group 5				
	289	292	311	
	281	278	302	
	273	274	295	
	268	265	292	
Mean	278	277	300	+ 23

## APPENDIX 7

EXPERIMENT 4—AVERAGE DAILY UREA AND AMMONIA NITROGEN  
EXCRETION (mg)

	Days 1-2	Days 3-4	Days 5-7	Days 8-10
Group 1			No burn	
	198	224	209	217
	232	208	304	293
	180	233	237	210
	158	213	226	194
	Mean	192	242	244
Group 2			Burn	
	162	249	209	
	176	221	252	248
	229	258	274	
	205	244	258	264 *
	Mean	193	243	263
	(190) †	(232)	(255)	(256)
Group 3			No burn + sucrose supplement	
	180	234	215	230
	168	208	157	194
	144	215	167	187
	191	234	184	208
	Mean	171	223	181
Group 4			Burn + sucrose supplement	
	192	224	175	182
	167	198	188	244
	198	241	207	241
	153	230	187	212
	186	225	183	220
	Mean	179	224	188
				226

\* This figure is calculated from the excretion on days 8 and 9 only. At the beginning of day 10 a feeding error necessitated that the animal be sacrificed.

† The figures in brackets are the means of the 2 animals that survived until day 10.

## APPENDIX 8

## EXPERIMENT 4 — ANIMAL WEIGHTS (g)

	Day 1	Day 5	Day 11	Change (days 5-11)
Group 1		No burn		
	296	291	293	
	287	277	273	
	270	256	255	
	269	264	265	
Mean	280	272	271	- 1
Group 2		Burn		
	293	277		
	275	268	253	
	262	253		
	264	258	248 *	
Mean	273 (270) †	264 (263)	(250)	- 13
Group 3		No burn +sucrose supplement		
	269	273	294	
	259	251	280	
	258	254	280	
	241	240	265	
Mean	257	254	280	+ 26
Group 4		Burn +sucrose supplement		
	293	279	327	
	267	267	288	
	257	244	262	
	250	238	265	
	290	276	310	
Mean	271	261	290	+ 29

\* This figure is the weight of the animal on the morning of day 10. At the beginning of day 10 a feeding error necessitated that the animal be sacrificed.

† The figures in brackets are the means of the 2 animals that survived until day 11.

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THE DETERMINATION OF THE RENAL CLEARANCE OF  
DIODONE AND THE MAXIMAL TUBULAR EXCRETORY  
CAPACITY FOR DIODONE IN MAN By J S ROBSON,  
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the Clinical Laboratory, Royal Infirmary, Edinburgh

(Received for publication 25th May 1949)

# INTRODUCTION

THE derivation of the renal clearance of inulin in man from the curve of the falling plasma inulin concentrations following a single intravenous injection [Robson, Ferguson, Olbrich and Stewart, 1949], suggested that a similar method of analysis might be applied to the determination of the renal clearance of diodone and to the maximal excretory capacity of the tubules for diodone

Homer Smith and his colleagues [1938] claimed that the renal clearance of diodone at plasma levels of less than 5 mg diodone iodine per 100 ml is a valid measure of the effective renal blood-flow, and that the maximal excretory power of the tubules for diodone, attained when the plasma level is elevated sufficiently to effect tubular saturation, provides a measure of the renal excretory mass. The difficulties of their technique, which involves the continuous infusion of diodone to maintain plasma levels first of less than 5 mg diodone iodine per 100 ml, and subsequently high enough to effect tubular saturation, the simultaneous infusion of inulin, and the collection of urine by catheter over short clearance periods, have rendered the method unsuitable for general clinical use

As in the case of the determination of the renal clearance of inulin,

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*Quart J exp Physiol*

## CORRIGENDUM SLIP TO PAPER BY G H LATHE AND R A PETERS

Vol 35, No 2, p 158 Table I  
For 173 read 167  
For - 32 read - 38

Vol 35, No 2, p 165 Exp 1, Group 2, Mean  
For 182 read 167



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As in the case of the determination of the renal clearance of inulin, there have been numerous attempts to simplify the technique. In all these, a single or double injection of diodone, variously given intravenously, subcutaneously or intramuscularly [Findley and White, 1940, Foà and Foà, 1942, Josephson, 1947, Barclay, Cooke and Kenney, 1947], replaces the continuous infusion. The collection of urine by catheter necessarily remains, with its inaccuracies and inconveniences, since the objective is an approximation to a value for the rate of excretion during an infinitesimally small period of time

Findley, Edwards, Clinton and White [1943] noted the impossibility of maintaining plasma concentrations of diodone high enough to effect

tubular saturation, following subcutaneous injection. Empirically, however, they claimed a high degree of correlation between the maximal tubular excretory capacity and the percentage of the injected diodone excreted in the first thirty minutes after injection. There appears to be no theoretical basis for this correlation, and, in our experience, the amount of diodone actually excreted in the first thirty minutes after injection has often deviated very widely from the amount predicted from a knowledge of the maximum tubular excretory power obtained by more orthodox methods. These discrepancies may arise partly from the fact that there is no constant relationship between the amount of diodone in the body at any given time, and the amount in the plasma presented to the kidneys for excretion.

Landowne and Alving [1946] have presented a method by which the rate of glomerular filtration, the renal blood-flow, and the maximal rate of tubular excretion were calculated from data obtained after a single injection of para-amino hippuric acid. Although this method appears to contain no theoretical fallacies in its application, the need for accurate urine collection by catheter remains. Earle and Berliner's method [1946] for determining the clearance of inulin and of diodone at low plasma levels, employing the infusion pump in order to maintain constancy in the plasma levels of these substances, has been briefly criticised elsewhere [Robson *et al.*, 1949], it has not, apparently, been applied to the determination of the maximal excretory power of the tubules for diodone.

## PART I VOLUME OF DISTRIBUTION

### *Theoretical Considerations*

In a previous paper, the present authors [1949] developed a method which, by taking cognisance of the continuously varying volume of distribution of inulin after injection, permitted the clearance of that substance to be measured simply. Similar considerations applied to diodone lead to methods of determining the renal clearance of diodone at low plasma levels ( $C_D$ ) and the maximal rate of tubular excretion of diodone at high plasma levels ( $Tm_D$ ).

Employing a terminology similar to that used on previous occasions [Dominguez, 1934, Dominguez and Pomerene, 1935, 1944, Robson *et al.*, 1949], the volume of distribution at any time is defined as that volume which would contain all the solute present in the body, if it were evenly distributed at the concentration found in plasma water at that time. The volume of distribution of diodone following a single injection of diodone may therefore be derived from the formula

$$V_D = \frac{D_s}{P}, \quad (1)$$

where  $V_D$  = the volume of distribution of diodone,

$D_b$  = the total amount of diodone in the body in mg diodone iodine,

$P$  = the concentration of diodone iodine in plasma water

By arguments similar to those used in the case of inulin, it may be shown that the function  $V_D$  does not necessarily possess any physiological significance in terms of actual volumes of defined fluid compartments

It may be shown that the function  $V_D = \frac{D_b}{P}$  provides a measure of the difference between the concentration of diodone in plasma water and the mean concentration in the fluids other than plasma in which it is dispersed. It also follows that the rate of change of the function  $V_D$  measures the change in the amount of diodone in all compartments other than plasma, relative to plasma water concentration, with respect to time

In the determination of  $V_D$ , values for  $D_b$  are calculated by subtracting the amount of diodone iodine excreted in the urine from the amount administered, at intervals following injection. It is clear that this method will yield true values for  $D_b$  only if diodone is excreted from the body solely by the kidneys, and provided that there is no destruction of it in the body. It is, however, by no means certain that these two conditions are fulfilled, although the amount of diodone which escapes renal excretion must be small. Tourné and Damm [1930] reported that a small quantity of sodium iodopyridone acetate was excreted by the liver, the amount so excreted increasing with diminishing renal efficiency. Exhaustive recovery experiments with diodone do not seem to have been reported, but in six human subjects investigated by the present authors, 77 to 100 per cent of intravenously injected diodone was recovered from the urine within five hours.

If diodone is destroyed in the body and/or excreted by any non-renal route, the value of  $D_b$  obtained by subtracting the amount of diodone iodine excreted in the urine from the amount originally injected will be greater than the true value of the amount of diodone iodine in the body. The values of  $V_D$  derived from such values of  $D_b$  must also be fictitious, and represent what, at given plasma diodone iodine levels, the volume of distribution would have been if renal excretion had been the only disposal route. Although this complicates the physiological significance of  $V_D$ , calculated from measurements of renal excretion of diodone, it does not prevent the development of a simplified method for determining the renal clearance of diodone or the maximal rate of tubular excretion for that substance.

## METHOD I

*The Determination of the Function  $V_D = \frac{D_b}{P}$* 

The function  $V_D = \frac{D_b}{P}$  following the intravenous injection of diodone was determined in fourteen subjects ranging in age from 25 to 83 years. Eleven of the subjects were old people suffering from varying degrees of hypertension but with no other complaint. The remaining three subjects were normal volunteers. With the exception of two normal female volunteers, who were permitted to be sedentary, the subjects were recumbent during the period of the experiment.

All fourteen subjects were employed in determining  $V_D$  with low plasma diodone levels, ten were given a second injection of diodone for the determination of  $V_D$  corresponding to high plasma levels, 60 to 80 minutes after the first, and two received the second injection after an interval of some days. In all cases inulin clearance was determined simultaneously with the determination of  $V_D$  for low plasma levels of diodone.

The subjects were prepared by fasting overnight, no breakfast was given and they were urged to drink ample amounts of fluid. To determine values of  $V_D$  corresponding to low plasma diodone levels, accurately measured volumes of a 35 per cent (w/v) solution of diodone were injected intravenously in approximately two minutes, for high plasma diodone levels a 50 per cent (w/v) solution was used and, the volumes being greater, the injection occupied about eight minutes. The times of the injection were reckoned from the mid-points of the actual injection period.

Venous blood samples were withdrawn at approximately twenty-minute intervals, for 55 to 120 minutes after injection when the low plasma diodone levels were being investigated, and for 87 to 129 minutes after the injection for the investigation of high plasma levels effecting tubular saturation. The normal female volunteers passed urine spontaneously at approximately twenty-minute intervals, errors of collection being minimised by maintaining a urine flow of over 10 ml/minute during the experimental period. In all other cases urine was collected by catheter, the bladder being washed out with 20 ml normal saline immediately before the end of each period. The exact time of every operation was noted. Diodone iodine was estimated in all blood and urine samples by Alpert's method [1941].

In all subjects, values of the function  $V_D = \frac{D_b}{P}$  following both injections were determined at intervals corresponding to times of urine collection. The total diodone iodine in the body at these times was

calculated by subtraction of the amount which had been excreted in the urine from the amount injected. In the ten subjects to whom the second larger injection was given 60 to 80 minutes after the first injection, the amount of diodone iodine remaining in the body from the initial injection at the time of the last urine collection was added to the amount given at the second injection. It was never more than 6 per cent of the second injection, which was invariably given within ten minutes of the preceding urine collection. The values of  $P$  employed in calculating  $V_D$  were derived from the plasma-time curve at the times of urine collection. They were converted to mg diodone iodine per 100 ml plasma water, using the formula  $\text{total plasma water per 100 ml plasma} = 100 - g \text{ plasma proteins per 100 ml plasma}$  [Goldring and Chasis, 1944]

## RESULTS I

Table I presents the clinical features of the subjects and the results of the values of  $V_D = \frac{D_0}{P}$  calculated at intervals following injection of diodone in amounts ranging from 18 to 36 mg diodone iodine/kg body-weight. These amounts produced plasma concentrations of diodone low enough to ensure high renal extraction ratios.

Table II gives the calculated values for  $V_D$  when, as a result of injecting 98 to 291 mg diodone iodine/kg body-weight, the plasma levels of diodone were high enough to ensure tubular saturation for the duration of the experiment. In this table the amount remaining in the body from any preceding injection is also noted.

In every subject,  $V_D$  increases throughout the experimental period. With the small dose of diodone,  $V_D$  varied from 10.0 to 28.8 l at times 18 to 38 minutes after injection, and from 13.0 to 61.5 l at times from 55 to 120 minutes. The initial values, when the larger injections were given, ranged from 7.5 to 19.5 l at times from 20 to 32 minutes after injection, and the final values, 87 to 129 minutes from the injection time, were 9.6 to 28.8 l.

Figs 1 and 2 illustrate graphically the relationship of  $V_D$  to time for some of the cases, the former for low plasma diodone levels, the latter for high. (Those cases which did not overlap on plotting were selected for the figures, the others do not differ in respect of the relationship of  $V_D$  to time.) Each line is drawn from the first value of  $V_D$  for the subject concerned (18 to 40 minutes after injection) to the final value for the same subject (58 to 130 minutes after injection), without consideration of the intermediate points which, however, fall close to the line.

As in the experiments reported for inulin (*loc cit*), the results indicate that at no time following the injection of diodone in doses

Subject	Sex	Age, Years	Weight, kg	B P	Diiodone iodine dose, mg /kg body-weight	Time in mins following injection	$V_D = \frac{D_0}{P}$ litres	
1	M.R.	F	25	55.5	120/70	29.5	20 40 60 80	23.2 33.4 44.6 61.5
2	J.S.	M	65	50.0	205/95	34.9	22 83 54 71	28.8 31.9 38.8 42.5
3	W.R.	M	49	71.1	160/110	24.5	38 54 70	26.0 28.8 30.3
4	M.F.	F	44	67.7	110/70	36.4	35 54 75 95 120	21.8 25.1 30.6 34.9 41.6
5	W.H.	M	44	56.8	140/90	30.5	21 41 60	17.5 24.4 32.5
6	R.D.	F	75	57.3	230/120	21.5	23 42 62	18.2 22.1 24.4
7	M.M.	F	72	50.5	250/140	18.3	18 38 58	10.0 11.9 13.0
8	C.B.	F	68	45.3	220/125	22.8	21 43 63	11.9 14.6 18.8
9	G.M.	M	70	58.5	190/110	26.7	22 41 62	19.5 23.5 28.5
10	M.L.	F	83	57.3	200/110	21.5	21 41 60	22.3 31.8 40.5
11	J.D.	M	78	67.5	220/130	18.4	22 40 55	17.5 20.0 21.6
12	B.J.	F	62	46.8	210/130	32.0	20 40 61	12.5 15.6 18.5
13	J.P.	F	70	39.1	220/120	26.6	21 41 61 81	11.1 13.0 14.1 15.6
14	B.D.	F	65	32.5	160/100	21.7	23 42 61	10.1 12.0 13.2

TABLE II—VALUES OF THE FUNCTION  $V_D = \frac{D_b}{P}$ , CALCULATED AT INTERVALS FOLLOWING INJECTION OF MEASURED AMOUNTS OF 50 PER CENT DIODONE, ADEQUATE TO PRODUCE PLASMA LEVEL SUITABLE FOR THE ESTIMATION OF  $Tm_D$ . THE AMOUNTS OF DIODONE REMAINING IN THE BODY FROM THE PREVIOUS INJECTIONS ARE INDICATED

Subject	Diodone iodine dose, mg /kg body weight	Diodone iodine in body from initial injection, mg /kg body weight	Time in mins following injection	$V_D = \frac{D_b}{P}$ litres
1 M R	291	0	20	16.7
			40	18.8
			60	21.0
			80	23.5
			100	26.0
			120	28.8
2 J S	249	10	32	19.5
			55	23.8
			80	27.2
			96	28.6
5 W.H.	252	9	24	12.5
			44	15.1
			63	16.7
			84	18.7
			105	19.6
			125	21.4
6 R.D	172	8	27	13.8
			45	15.4
			65	16.9
			86	18.2
			105	19.2
			125	20.3
7 M.M	172	5	28	12.4
			49	15.0
			68	16.7
			77	17.5
			108	19.2
8 C.B	203	9	25	10.3
			44	12.0
			64	14.1
			84	15.9
			104	17.1
9 G.M	211	10	30	13.8
			49	16.0
			69	18.5
			89	19.0
			109	19.9
10 M.L	215	12	25	12.4
			46	14.3
			67	15.6
			86	16.6
			106	17.4
			128	18.1

TABLE II—*Continued*

Subject	Diodone iodine dose, mg /kg body weight	Diodone iodine in body from initial injection, mg /kg body weight	Time in mins following injection	$V_D = \frac{D_s}{P}$ litres
11 J D	98	0	22	19 0
			32	20 0
			43	20 7
			87	23 2
12 B J	197	11	29	10 8
			49	12 5
			69	13 5
			88	14 2
			109	14 8
13 J P	240	10	129	15 5
			29	9 2
			51	10 3
			71	11 1
			92	11 9
14 B D	227	9	114	12 3
			26	7 5
			46	8 5
			65	8 0
			86	9 4
			106	9 2
			126	9 6

of 18 to 36 mg /kg body-weight or 98 to 291 mg /kg body-weight does the function  $V_D$  attain a constant value. The implications of this finding in attempts to derive values for renal clearance of diodone and maximal tubular excretory capacity for diodone from curves relating the falling plasma concentrations to time, will be developed in the theoretical discussion which follows.

## PART II THE RENAL CLEARANCE AND THE MAXIMAL TUBULAR EXCRETORY CAPACITY FOR DIODONE

### *Theoretical Considerations*

When diodone is injected into the blood stream, part passes out of the blood-vessels and is distributed in the body fluids. There is a disparity of opinion with regard to the entry of diodone into the red blood cells. Smith and his colleagues [1938], from *in vitro* experiments, claimed that these cells were impermeable to it. More recently, experiments by White, Findley and Edwards [1940] have indicated that, following the intravenous administration of diodone the concentration in arterial cell water is about 30 per cent of the plasma concentration. Irrespective of the method employed in the derivation of the clearance



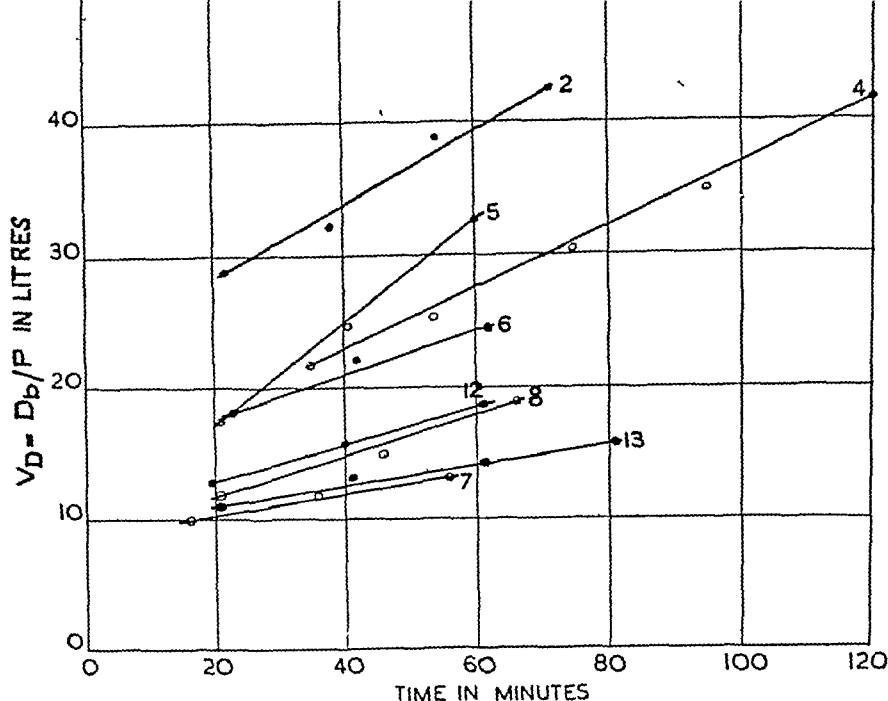


FIG. 1—Values of the function  $V_D = \frac{D_b}{P}$  plotted against time for subjects Nos 2, 4, 5, 6, 7, 8, 12 and 13. Each line is drawn from the first value of  $V_D$  for the subject to the final value determined for the same subject without consideration of the intermediate points. Diodone was given in amounts ranging from 18 mg to 36 mg diodone iodine/kg body weight at time 0.

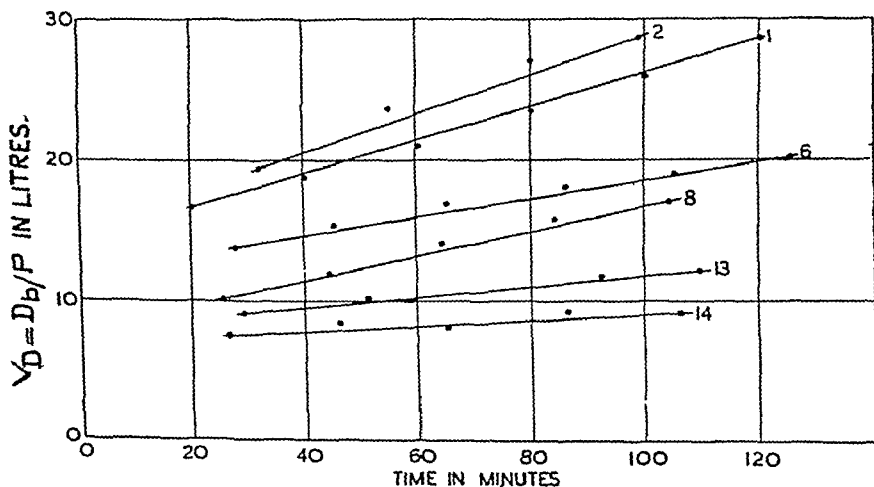


FIG. 2—Values of the function  $V_D = \frac{D_b}{P}$  plotted against time for subjects Nos 1, 2, 6, 8, 13 and 14. Each line is drawn from the first value of  $V_D$  for the subject to the final value determined for the same subject without consideration of intermediate points. Diodone was given in amounts ranging from 98 mg to 291 mg diodone iodine/kg body weight at time 0.

of diodone at low plasma levels ( $C_D$ ), and that of the maximal excretory power of the tubules for diodone ( $Tm_D$ ), the possibility of entry into the blood cells is of importance only if it contributes significantly to the plasma concentration during circulation through the kidneys and thereby adds to the urinary diodone. The results obtained by White, Findley and Edwards [1940] indicate that this contribution is certainly small. Future information may show the desirability of introducing a correction for any addition to the urinary diodone by the circulating cells, but here the normal practice will be adopted of assuming that only plasma diodone is filtered by the glomeruli and excreted by the tubules during passage through the kidneys. The possibility of the entry of diodone into tissue cells other than red cells is irrelevant to the theoretical considerations which follow.

Smith and his colleagues [1938] claim that at plasma concentrations below a specified level, diodone is excreted by the kidneys at a rate proportional to the plasma level. It is further stated that the renal clearance of diodone at these levels represents the effective renal plasma flow [Smith, Goldring and Chasis, 1938, Smith, 1943]. These relationships may be expressed as follows —

$$\frac{dD_b}{dt} = -U_D V = -C_D' P,$$

where  $U_D V$  = the rate of excretion of diodone iodine in the urine,

$C_D'$  = a constant, representing the renal clearance of diodone in terms of plasma water,

$P$  = the plasma water concentration of diodone, as mg diodone iodine

By definition,

$$\begin{aligned} D_b &= P \times V_D \\ \frac{dD_b}{dt} &= \frac{d(P \times V_D)}{dt} \\ &= P \frac{dV_D}{dt} + V_D \frac{dP}{dt} \\ -C_D' &= \frac{dV_D}{dt} + \frac{V_D}{P} \frac{dP}{dt} \end{aligned} \quad (2)$$

Equation (2) has a form similar to the basic formula developed for inulin by Robson, Ferguson, Olbrich and Stewart [1949]. The equation may be solved, provided the simplifying assumption is made that, between definable limits of time,  $V_D$  is linearly related to time. Fig 1 suggests that this is justified as an approximation, but the magnitude of the error involved will be discussed later. For the purpose of further

analysis, therefore, the function  $V_D$  will be expressed as a straight line

$$V_D = p + qt$$

Equation (2) becomes

$$\frac{p + qt}{P} \frac{dP}{dt} = -(C_D' + q)$$

This equation may be solved by a procedure identical with that adopted for inulin (*loc cit*), and the intermediate steps need not, therefore, be repeated. The final formulæ which emerge are as follows —

$$P = K' V_D^{-\frac{(C_D' + q)}{q}} \quad (3)$$

and

$$C_D' = \frac{q (\log P_1 - \log P_2)}{\log (V_D)_2 - \log (V_D)_1} - q, \quad (4)$$

where  $K'$  = a constant,

$q$  = a constant representing the slope of the line  $V_D = p + qt$ ,

$C_D'$  = the renal clearance of diodone in terms of plasma water,

$P_1$  = the plasma water concentration of diodone iodine at time  $t_1$ ,

$P_2$  = the plasma water concentration of diodone iodine at time  $t_2$ ,

$(V_D)_1$  = the volume of distribution of diodone at time  $t_1$ ,

$(V_D)_2$  = the volume of distribution of diodone at time  $t_2$ .

The logarithms are to the base 10,  $t_1$  and  $t_2$  are times within which it is assumed that the function  $V_D$  is linearly related to time.

The method of applying formula (4) to the determination of the renal clearance of diodone, and the results obtained by its use, will be discussed in the next section. It is clear that the calculative error in the use of this formula lies in the assumption of linearity in the relationship of the function  $V_D$  to time. As in the case of inulin, the magnitude of the error so introduced may be represented by the deviations of values of  $P$  on the smooth curve drawn through experimentally determined points from those obtained employing formula (3).

### *The Derivation of a Formula for the Measurement of the Maximal Tubular Excretory Capacity*

At plasma levels of diodone high enough to effect tubular saturation, diodone is excreted by the kidneys at a rate which is claimed to be the sum of the rate of glomerular filtration and the maximal rate of tubular excretion [Smith, Goldring and Chasis, 1938]. This relationship is expressed as follows, using the symbols employed by Smith and his colleagues [1938] and by Goldring and Chasis [1944] —

$$Tm_D = U_D V - FWC_{in} P_D, \quad (5)$$

where  $Tm_D$  = maximal tubular excretory capacity for diodone in mg /minute,

$U_D V$  = rate of excretion of diodone iodine in mg /minute,

$F$  = the fraction of diodone that is free in plasma water and therefore filterable [Smith and Smith, 1938],

$W$  = the fraction of water in plasma,

$P_D$  = plasma concentration of diodone iodine in mg /ml  
plasma =  $WP$ ,

$C_{in}$  = rate of glomerular filtration in ml /minute, as measured by the renal clearance of inulin

The rate of excretion of diodone iodine ( $U_D V$ ) at such plasma levels is no longer proportional to the plasma level, and the simple formulæ derived for the measurement of the renal clearances of diodone at low plasma concentrations and of inulin are no longer applicable

Formula (5) may be rearranged thus

$$U_D V = Tm_D + FWC_{in}P_D$$

This may be expressed

$$\frac{dD_b}{dt} = -(Tm_D + FWC_{in}P_D)$$

The negative sign is introduced to indicate that the amount of diodone in the body decreases with time

By definition,

$$D_b = P \times V_D$$

$$\frac{dD_b}{dt} = \frac{d}{dt}(P \times V_D)$$

$$= P \frac{dV_D}{dt} + V_D \frac{dP}{dt}$$

$$P \frac{dV_D}{dt} + V_D \frac{dP}{dt} = -(Tm_D + FWC_{in}P_D)$$

$$Tm_D + FWC_{in}P_D + P \frac{dV_D}{dt} = -V_D \frac{dP}{dt}$$

By definition,

$$P_D = W P$$

$$Tm_D + \left( FW^2 C_{in} + \frac{dV_D}{dt} \right) P = -V_D \frac{dP}{dt} \quad (6)$$

Equation (6) may be solved, provided the simplifying assumption is made that between definable limits of time the function  $V_D = \frac{D_b}{P}$

is linearly related to time Fig 2 suggests this assumption is justified as an approximation, but the magnitude of the error introduced will be discussed later For the purpose of further analysis, therefore, the function  $V_D$  can be expressed as a line

$$V_D = l + mt,$$

where  $m$  and  $l$  are constants of the line

For convenience in the analysis, the function  $FW^2C_{in}$  will be represented by  $C'$  Its constancy will of course be assumed

Formula (6) then becomes

$$Tm_D + (C' + m) P = -(l + mt) \frac{dP}{dt}$$

Solving this equation for  $P$ , we have

$$\int \frac{dP}{Tm_D + (C' + m)P} = - \int \frac{dt}{l + mt},$$

$$\frac{\ln [Tm_D + (C' + m)P]}{C' + m} = - \frac{\ln (l + mt)}{m} + \frac{\ln K}{C' + m}$$

where  $\frac{\ln K}{C' + m}$  is the constant of integration

$$\ln [Tm_D + (C' + m)P] = \ln K(l + mt)^{-\frac{(C' + m)}{m}}$$

$$Tm_D + (C' + m)P = K(l + mt)^{-\frac{(C' + m)}{m}} \quad (7)$$

$$P = \frac{K V_D^{-\frac{(C' + m)}{m}} - Tm_D}{C' + m} \quad (8)$$

It is clear that equation (7) may be solved for  $Tm_D$ , given two plasma water diodone iodine concentrations,  $P_1$  and  $P_2$ , determined at times  $t_1$  and  $t_2$  within the limits of time during which it has been assumed that  $V_D$  is linear with time

Let  $(V_D)_1$  and  $(V_D)_2$  equal the function  $\frac{D_b}{P}$  at times  $t_1$  and  $t_2$

From equation (7),

$$Tm_D + (C' + m)P = K V_D^{-\frac{(C' + m)}{m}}$$

$$\log [Tm_D + (C' + m)P_1] = \log K - \frac{C' + m}{m} \log (V_D)_1$$

Similarly

$$\log [Tm_D + (C' + m)P_2] = \log K - \frac{C' + m}{m} \log (V_D)_2$$

By subtraction,

$$\begin{aligned}\log \frac{Tm_D + (C' + m)P_1}{Tm_D + (C' + m)P_2} &= \frac{C' + m}{m} \log \frac{(V_D)_2}{(V_D)_1} \\ Tm_D + (C' + m)P_1 &= [Tm_D + (C' + m)P_2] \left\{ \frac{(V_D)_2}{(V_D)_1} \right\}^{\frac{C' + m}{m}} \\ Tm_D \left[ 1 - \frac{(V_D)_2}{(V_D)_1}^{\frac{C' + m}{m}} \right] &= (C' + m) \left[ P_2 \left\{ \frac{(V_D)_2}{(V_D)_1} \right\}^{\frac{C' + m}{m}} - P_1 \right] \\ Tm_D \left[ (V_D)_1^{\frac{C' + m}{m}} - (V_D)_2^{\frac{C' + m}{m}} \right] &= (C' + m) \left[ P_2 (V_D)_2^{\frac{C' + m}{m}} - P_1 (V_D)_1^{\frac{C' + m}{m}} \right] \\ Tm_D &= (C' + m) \frac{P_2 (V_D)_2^{\frac{C' + m}{m}} - P_1 (V_D)_1^{\frac{C' + m}{m}}}{(V_D)_1^{\frac{C' + m}{m}} - (V_D)_2^{\frac{C' + m}{m}}} \quad (9)\end{aligned}$$

The calculative error in the determination of the tubular excretory capacity, employing formula (9), is solely the error introduced by the assumption of the linearity of  $V_D$  with time. The error may be represented by deviations of the values of  $P$  determined by the use of formula (8) from those values of  $P$  obtained from the smooth curve constructed through experimentally determined plasma levels.

This derivation of the formulae for the renal clearance of diodone and the tubular excretory capacity involves the differentiation of the function  $D_b$  with respect to time in terms of the product of  $P$  and  $V_D$ . The values for  $V_D$  are obtained from values for  $D_b$  which result when the amount of diodone excreted in the urine is subtracted from the amount originally injected. It has already been indicated that this process does not necessarily produce true values for the amount of diodone in the body at any time, because the possibility of hepatic excretion or destruction of diodone in the body cannot be excluded. The criticism may, therefore, be offered that the differentiation of  $D_b$  with time, employing these values for  $V_D$ , is not a valid one, since the rate of change of diodone in the body may actually be greater than that accounted for solely by renal excretion.

That this criticism is not valid follows from the fact that although  $D_b$  is defined as the amount of diodone in the body at any time, the method of calculating  $D_b$  provides values which would occur only if the kidneys were the sole excretory route, and there was no destruction in the body. The rate of change with respect to time of this function will therefore represent the rate of renal excretion. Since by definition  $D_b = PV_D$ , the expression  $\frac{d(PV_D)}{dt}$  also represents the excretion rate

## METHODS II

*The Determination of the Renal Clearance of Diodone and the Maximal Tubular Excretory Capacity for Diodone*

*The Experimental Determination of  $C_D$  and  $Tm_D$* —The renal clearance of diodone was determined in all fourteen subjects, using the data obtained for the determination of the function  $V_D$  and utilised in constructing Table I. Values for renal clearance following the intravenous injection of diodone were obtained for two to five periods of about 20 minutes each. For each period, the clearance of diodone was determined by dividing the mean rate of diodone iodine excretion per minute by the plasma level, the latter being read from the curve of plasma diodone iodine values plotted against time. The concentration selected was one occurring  $2\frac{1}{2}$  minutes before the mid-point of the clearance period. As with inulin, the value of  $P$  selected in this way may be shown not to differ significantly from the one obtained by estimating the area subtended by the plasma diodone iodine-time curve between the limits of time  $2\frac{1}{2}$  minutes before the beginning and  $2\frac{1}{2}$  minutes before the end of the clearance period and dividing by the abscissa. Although urine was collected approximately every 20 minutes after injection, no clearance value was determined earlier than 20 minutes after the injection.

In the derivation of values for  $C_D$  by this method, the mean values of plasma diodone iodine employed for all periods were less than 5 mg/100 ml plasma, for all cases except cases Nos 12 and 13, in whom the values of  $P$  used for the first period were 6.0 mg and 5.5 mg/100 ml plasma respectively. Since, however, the estimations were made in subjects in whom the plasma concentration of diodone iodine was falling, the concentration during the first few minutes of the first period was above 5.0 mg/100 ml in six of the cases studied, extending to a maximum of 7.8 mg/100 ml at the beginning of the first period in case No 12. On the basis of the standards set by Smith and his colleagues [1938], the clearance of diodone would be expected to be depressed at these higher levels, though the extent of the depression would be small. In actuality, no evidence of depression of clearance occurs in any of these cases, nor indeed was there any evidence of self-depression in five other subjects in whom determinations of diodone clearance were made with plasma levels extending up to 10 mg/100 ml plasma following a single injection of diodone. This finding is in general agreement with that of White, Findley and Edwards [1940].

The maximal tubular excretory capacity for diodone was measured in twelve subjects to whom a second larger injection of diodone was given, the data used in the construction of Table II being employed. Beginning 18 to 38 minutes after injection, values for  $Tm_D$  were obtained for

3 to 5 subsequent periods of about 20 minutes. The values were obtained by subtracting the estimated average amount of diodone filtered by the glomeruli from the average excreted in the urine per minute for the period, according to formula (5). The rate of glomerular filtration was estimated by measuring the renal clearance of inulin by the authors' method [1949], for the period over which urine was collected for  $C_D$  and  $Tm_D$  estimations. In addition, the collection of urine every 20 minutes permitted the derivation of values for inulin clearance for each clearance period over the period of urine collection by the orthodox process of dividing the average rate of renal excretion of inulin per minute by the mean plasma level of inulin for the period. This permitted an extension of the series of subjects in whom comparison of values for inulin clearance determined by the method devised by the authors (*loc cit*) could be made with the average value for inulin clearance determined by the direct experimental method. For these purposes, the inulin was injected intravenously immediately after the first injection of diodone in all subjects.

In the derivation of values for  $Tm_D$  by this method, care was taken to ensure that saturation of the tubules with diodone, as defined by Smith [1943] and by Goldring and Chasis [1944], was effected for the duration of the experiment. The load/ $Tm_D$  ratio during the last period in which  $Tm_D$  was determined was not less than 2.0 in all subjects, except in cases Nos 7, 8 and 11 in whom the ratio was not less than 1.5.

The values obtained for the renal clearance of diodone and maximal tubular excretion by these methods will be termed "experimentally determined values" for  $C_D$  and  $Tm_D$ , in contradistinction to the "calculated values" obtained by using the formulæ developed in this paper.

#### *The Determination of the Renal Clearance of Diodone Employing Formula (4)*

Between the times  $t_1$  and  $t_2$ , stated in each case, and approximately 20 to 70 minutes respectively after injection, the function  $V_D$  was assumed to be linearly related to time. The times  $t_1$  and  $t_2$  corresponded to times of urine collection, and values of  $V_D$  at these times were obtained by the method already described. Values of  $P_1$  and  $P_2$  were obtained from the plasma diodone iodine-time curve. They were expressed in terms of mg diodone iodine/100 ml plasma water. The constants of the line  $V_D = p + qt$  between the points  $t_1, (V_D)_1$  and  $t_2, (V_D)_2$ ,  $p$  and  $q$ , were calculated for each case. Introducing the values of  $q, (V_D)_1$  and  $(V_D)_2$  into formula (4), a value for  $C'_D$  was derived for each case. If the values for  $(V_D)_1$  and  $(V_D)_2$  are expressed in terms of hundreds of ml, values of  $C'_D$  obtained by the formula may be converted to values which



represent the plasma clearance of diodone per minute ( $C_D$ ) by multiplication of the values for  $C_D'$  by the factor

$$\frac{10,000}{100 - \text{g plasma protein}/100 \text{ ml}}$$

For ordinary purposes, the plasma renal clearance may be taken as  $C_D' \times 106$

*The Determination, by the Use of Formula (3), of the Error introduced in the Assumption of Linearity of the Function  $V_D$  with Time, between  $t_1$  and  $t_2$ , following the Injection of Diodone in Amounts adequate for the Measurement of  $C_D$*

The procedure is similar to that described for inulin (*loc cit*) Equation (3) may be solved for  $K'$  by substituting an experimentally determined value for  $P$ , i.e.  $P_1$  at time  $t_1$ , and using (1) values of  $q$  calculated from line  $V_D = p + qt$ , between the points  $t_1, (V_D)_1$  and  $t_2, (V_D)_2$ , and (2) the value of  $C_D'$  obtained by the use of formula (4) Employing this calculated value for  $K'$ , values of  $P$  at varying values of  $t$  are obtained. The extent to which these derived values of  $P$  differ from those given by the graph constructed on the basis of experimentally determined values, is a measure of the error involved in the use of formulæ (3) and (4), i.e. in the assumption that the function  $V_D = \frac{D_0}{P}$  is linear between the time-limits selected

*The Determination of the Maximal Tubular Excretory Capacity for Diodone Employing Formula (9)*

Between the times  $t_1$  and  $t_2$  stated for each case, and approximately 20 and 120 minutes respectively after the injection of diodone in amounts sufficient to saturate the tubules, the function  $V_D$  is again assumed to be linearly related to time The times  $t_1$  and  $t_2$  correspond to the times of urine collection, and values of the function  $V_D$  at these times were obtained by the method already described Values of  $P_1$  and  $P_2$  were derived from the plasma diodone iodine-time curve They were expressed in mg/100 ml plasma water The constants of the line  $V_D = l + mt$ , between the points  $t_1, (V_D)_1$  and  $t_2, (V_D)_2$ , were calculated for each case Introducing the values for  $m, P_1$  and  $P_2, (V_D)_1$  and  $(V_D)_2$ , and for  $C'(FW^2C_{in})$ , into formula (9), a value for  $Tm_D$  was derived for each case If the values for  $V_D$  are expressed in hundreds of ml, the values for  $P_1$  and  $P_2$  in mg/100 ml plasma water and the value for  $C'$  in hundreds ml/minute, values for  $Tm_D$  are obtained directly in terms of mg per minute

*The Determination, by the Use of Formula (8), of the Error involved in the Assumption of Linearity in the Function  $V_D$  between the Times  $t_1$  and  $t_2$ , following the Injection of Diodone in Amounts Sufficient to Effect Tubular Saturation*

Equation (8) may be solved for  $K$  by substituting experimentally determined values for  $P_1$  and  $P_2$  at times  $t_1$  and  $t_2$ , and using (1) values of  $m$  calculated from the line  $V_D = l + mt$ , between the points  $t_1, (V_D)_1$  and  $t_2, (V_D)_2$ , (2) the values of  $Tm_D$  obtained by the use of formula (8), and (3) a value for  $C'$  obtained from an estimation of the renal clearance of inulin. Employing this calculated value for  $K$ , values for  $P$  at varying values of  $t$  are obtained. The extent to which these derived values of  $P$  differ from those given by the graph constructed on the basis of experimentally determined values, is a measure of the error introduced in the use of formula (8) by the assumption that the function  $V_D = \frac{D_0}{P}$  is linear between the limits of time selected.

## RESULTS II

Table III presents the average experimental plasma clearance of inulin for thirteen subjects, the values ranging from 51 to 112 ml /minute, and compares them with values derived by the application of the formula for inulin clearance described by the authors (*loc cit*)

TABLE III—COMPARISON OF VALUES FOR THE RENAL CLEARANCE OF INULIN EXPERIMENTALLY DETERMINED WITH THOSE CALCULATED ACCORDING TO THE METHOD OF THE AUTHORS [1949]

Subject	Experimental plasma clearance, ml /min Average of 3-6 periods	Calculated plasma clearance, ml /min
1 M R *	90	88
2 J S	112	
3 W.R		
4 M.F *	81	80
5 W H *	92	89
6 R.D	65	65
7 M M *	79	81
8 C B	57	56
9 G.M.	78	80
10 M.L	76	77
11 J.D	43	46
12 B J	65	66
13 J P	47	48
14 B.D	51	52

\* Reported previously [1949]

The difference between the value for clearance calculated and the average value obtained from 3 to 5 short clearance periods in which

urine was collected by catheter approximately every 20 minutes, is never more than  $\pm 3$  ml/minute

Table IV presents the results of the experimentally determined renal plasma clearances of diodone. The results of the individual clearance periods and the average for the series are shown in each case. The average plasma clearance ranges from 170 to 558 ml/minute.

TABLE IV — EXPERIMENTALLY DETERMINED VALUES FOR THE PLASMA RENAL CLEARANCE OF DIODONE

Subject	Experimental clearance at each clearance period, ml/min	Average experimental clearance, ml/min
1 M R	452	418
	400	
	402	
2 J S	680	558
	514	
	479	
3 W R	572	500
	486	
	442	
4 M F	351	306
	297	
	291	
	284	
5 W H	463	441
	419	
6 R D	305	278
	250	
7 M M	228	220
	212	
8 C B	200	183
	166	
9 G M	430	374
	317	
10 M L	270	334
	398	
11 J D	175	180
	186	
12 B J	303	282
	260	
13 J P	181	155
	153	
	132	
14 B D	170	170
	170	

The experimentally determined values for  $Tm_D$  are shown in Table V. The values for individual clearance periods and the average for the series are shown for each subject. The average values range from 4.7 to 36.3 mg diodone iodine per minute.

The values for the plasma clearance of diodone employing formula (4) are shown in Table VI. The limits of time,  $t_1$  and  $t_2$ , between which  $V_D$  was assumed to be linear with time, are recorded, as are the values for  $(V_D)_1$  and  $(V_D)_2$  at those times, and the constants of the line  $V_D = p + qt$ . The table includes plasma water diodone iodine levels,  $P_1$  and  $P_2$ , derived from the plasma diodone iodine-time curve drawn through experimentally determined points. The values for  $C_D'$ , derived by the use of the formula, are given, and the results of the conversion of  $C_D'$  to actual values of plasma clearance. For convenience in comparison, the averages of the experimentally determined values (Table IV) for plasma clearance for the subjects are shown, in brackets, in column 7 after the corresponding calculated value. The mean percentage difference between the two series of results for plasma clearance is -3.6, with a standard deviation of  $\pm 3.9$ .

Table VII presents the results for  $Tm_D$ , calculated by formula (9). The limits of time,  $t_1$  and  $t_2$ , between which  $V_D$  was assumed to be linear with time are shown, as are the values for  $(V_D)_1$  and  $(V_D)_2$  at these times, and the constants of the line  $V_D = l + mt$ . Values for  $C'(FW^2C_{in})$  are given, and the plasma water concentrations,  $P_1$  and  $P_2$ , at times  $t_1$  and  $t_2$ , respectively. Column 7 includes the calculated values for  $Tm_D$  and the corresponding average value for  $Tm_D$ , determined by the direct experimental method. The correspondence between the two values is excellent. The maximum difference between the two series of results is 1.6 mg diodone iodine per minute (5 per cent of the experimental value).

Results obtained in calculating plasma-diodone concentrations by the use of formulæ (3) and (8) are shown in Tables VIII and IX respectively. Values of plasma water diodone iodine in mg/100 ml following the injection of diodone for  $C_D$  determinations, taken from curves constructed through values experimentally determined, at about twenty-minute intervals, are compared with values for plasma water diodone iodine calculated by the use of formula (3). The differences between the results,  $\Delta$ , are given. In each case, the time when the formula was fitted is indicated. In no case is the deviation of the calculated from the experimental value greater than 0.1 mg/100 ml plasma water, and in several cases there is complete correspondence between the values.

In Table IX a similar comparison is made for the high plasma water diodone iodine concentrations utilised for determination of  $Tm_D$ , using formula (8). The formula was fitted to the diodone time curves approximately 20 to 30 minutes after injection, the exact time being

TABLE V—EXPERIMENTALLY DETERMINED VALUES FOR MAXIMAL TUBULAR EXCRETORY CAPACITY FOR DIODONE

Subject	Experimental $T_{md}$ at each clearance period,	Experimental $T_{md}$ Average
	mg diodone iodine/minute	mg diodone iodine/minute
1 M.R.	33.3	
	34.0	
	38.6	
	39.7	
	35.6	36.3
2 J.S.	27.2	
	31.4	
	34.7	
	31.2	31.1
5 W.H.	24.5	
	35.7	
	28.0	
	32.6	
	24.7	29.1
6 R.D.	30.9	
	23.5	
	24.5	
	23.9	
	22.5	25.1
7 M.M.	25.7	
	24.7	
	28.0	
	20.4	24.7
8 C.B.	23.6	
	19.5	
	21.8	21.7
9 G.M.	19.3	
	20.1	
	24.9	
	23.2	21.9
10 M.L.	23.4	
	21.0	
	18.6	
	17.4	
	16.0	19.3
11 J.D.	19.3	
	19.2	
	19.6	
	12.7	19.4
12 B.J.	15.1	
	15.0	
	14.1	
	13.5	
	10.6	14.1
13 J.P.	11.6	
	10.2	
	9.3	
	9.1	10.4
14 B.D.	5.1	
	3.7	
	3.0	
	2.4	
		4.7

TABLE VI—CALCULATION OF PLASMA DIODONE CLEARANCE  $C_{D'} = \frac{q(\log P_1 - \log P_2)}{\log(VD)_2 - \log(VD)_1} - q$ 

1	2	3	4	5	6	7
Subject	$t_1$ (mins)	$(VD)_1$ in 100 ml	$P$	$P_1$ mg per 100 ml plasma water	$C_{D'}$	Plasma clearance diodone ml per min $C_{D'} \times \frac{10,000}{100 - \text{g plasma protein per 100 ml plasma}}$
1 M R	20 80	232 615	104 3	5 1 1 0	4 284	456 (418)
2 J S	22 71	288 425	226 5	3 7 1 2	5 293	563 (558)
3 W R	36 70	260 303	208 0	2 3 1 2	4 373	470 (500)
4 M F	35 120	216 416	137 6	6 2 1 4	3 035	326 (306)
5 W H	21 60	175 325	94 2	0 0 1 0	4 300	409 (441)
6 R D	23 62	182 244	145 4	4 2 1 0	2 712	289 (278)
7 M M	18 58	100 130	80 5	4 8 1 8	2 054	219 (220)
8 C B	21 63	110 188	84 5	5 0 2 1	1 879	200 (183)
9 G M	22 62	195 285	145 5	5 8 2 1	3 774	307 (374)
10 M L	21 60	223 405	125 0	4 7 1 7	3 285	353 (334)
11 J D	22 55	175 216	147 7	5 7 3 5	1 033	174 (180)
12 B J	20 61	128 185	100 2	8 2 2 7	2 801	295 (282)
13 J P	21 81	111 156	95 3	7 2 2 6	1 494	161 (155)
14 B D	23 61	101 132	82 2	4 8 2 1	1 7024	176 (170)

For convenience in comparison, the average values for the experimentally determined clearances, corresponding to the values obtained by use of the formula, are shown in brackets in column 7

TABLE VII—CALCULATION OF TUBULAR EXCRETORY CAPACITY FOR DIODONE

1	2	3	4	5	6	7
Subject	$t_1$ $t_2$ (mins)	$(Vd)_1$ in 100 ml	$l$ $n$	$C' = F W^2 C_{in}$ in 100 ml per min	$P_1$ $P_2$ mg per 100 ml plasma water	$T^{md}$ mg diiodone iodine per min
1 MR	20	167	142.8	0.5909	85.1	37.0 (36.3)
2 JS	32	195	149.5	0.7685	50.0	31.5 (31.1)
5 WH	24	125	103.8	0.5894	97.8	28.2 (29.1)
6 RD	27	138	120.0	0.4516	62.2	25.0 (25.1)
7 MM	28	124	100.2	0.5498	56.4	24.1 (24.7)
8 CB	25	103	81.48	0.3874	76.1	21.0 (21.7)
9 GM	30	138	114.8	0.5812	79.0	21.0 (21.9)
10 ML	25	124	110.2	0.5101	91.4	20.1 (19.3)
11 JD	22	190	175.7	0.6462	33.5	18.3 (19.4)
12 BJ	20	108	94.37	0.4632	76.8	14.7 (14.1)
13 JP	29	92	81.4	0.3288	94.6	9.9 (10.4)
14 BD	26	75	69.5	0.3725	86.8	4.5 (4.7)

For convenience in comparison, the average values for the experimentally determined clearances, corresponding to the values obtained by use of the formula, are shown in brackets in column 7

noted in each case. At times later than 20 to 30 minutes after injection, the differences between the two series of values were never greater than +4.7 mg/100 ml plasma water, and were usually much less. At times earlier than 20 minutes after injection, the maximum difference reached 16 mg/100 ml plasma water. The limits of error in the chemical determination of diodone in plasma were found to be  $\pm 5$  per cent ( $P=0.99$ ).

TABLE VIII—COMPARISON OF VALUES OF THE PERCENTAGE CONCENTRATION OF DIODONE IODINE IN PLASMA WATER DERIVED FROM THE BEST CURVE DRAWN THROUGH EXPERIMENTALLY DETERMINED POINTS WITH THOSE CALCULATED ACCORDING TO THE FORMULA

$$P = K'V_D \frac{(C_D + g)}{g}$$

THE AMOUNT OF DIODONE INJECTED IN ALL CASES WAS ADEQUATE TO ESTABLISH PLASMA CONCENTRATIONS SUITABLE FOR MEASUREMENTS OF RENAL PLASMA-FLOW

Subject		<i>t</i>	20 ( <i>f</i> )	40	60	80	
1 M.R.		<i>t</i>	20 ( <i>f</i> )	40	60	80	
	Experimental	<i>P</i>	5.1	2.6	1.6	1.0	
	Calculated	<i>P</i>	5.1	2.5	1.5	1.0	
		$\Delta$	0	- 0.1	- 0.1	0	
2 J.S.		<i>t</i>	22 ( <i>f</i> )	38	54	71	
	Experimental	<i>P</i>	3.7	2.3	1.6	1.2	
	Calculated	<i>P</i>	3.7	2.4	1.7	1.2	
		$\Delta$	0	+ 0.1	+ 0.1	0	
3 W.R.		<i>t</i>	38 ( <i>f</i> )	54	70		
	Experimental	<i>P</i>	2.3	1.6	1.2		
	Calculated	<i>P</i>	2.3	1.6	1.2		
		$\Delta$	0	0	0		
4 M.F.		<i>t</i>	35 ( <i>f</i> )	54	75	95	120
	Experimental	<i>P</i>	6.2	4.1	2.7	2.0	1.4
	Calculated	<i>P</i>	6.2	4.0	2.7	2.0	1.4
		$\Delta$	0	- 0.1	0	0	0
5 W.H.		<i>t</i>	21 ( <i>f</i> )	41	60		
	Experimental	<i>P</i>	6.0	2.8	1.6		
	Calculated	<i>P</i>	6.0	2.8	1.6		
		$\Delta$	0	0	0		
6 R.D.		<i>t</i>	23 ( <i>f</i> )	42	62		
	Experimental	<i>P</i>	4.3	2.6	1.9		
	Calculated	<i>P</i>	4.2	2.7	1.9		
		$\Delta$	0	+ 0.1	0		
7 M.M.		<i>t</i>	18 ( <i>f</i> )	38	58		
	Experimental	<i>P</i>	4.8	2.7	1.8		
	Calculated	<i>P</i>	4.8	2.8	1.8		
		$\Delta$	0	+ 0.1	0		
8 C.B.		<i>t</i>	21 ( <i>f</i> )	43	63		
	Experimental	<i>P</i>	5.6	3.3	2.1		
	Calculated	<i>P</i>	5.6	3.2	2.1		
		$\Delta$	0	- 0.1	0		



TABLE VIII—Continued

9 G.M	<i>t</i>	22 ( <i>f</i> )	41	62	
Experimental	<i>P</i>	5.8	3.3	2.1	
Calculated	<i>P</i>	5.8	3.4	2.1	
	$\Delta$	0	+ 0.1	0	
10 M.L	<i>t</i>	21 ( <i>f</i> )	41	60	
Experimental	<i>P</i>	4.7	2.7	1.7	
Calculated	<i>P</i>	4.7	2.6	1.7	
	$\Delta$	0	- 0.1	0	
11 J.D	<i>t</i>	22 ( <i>f</i> )	40	55	
Experimental	<i>P</i>	5.7	4.3	3.5	
Calculated	<i>P</i>	5.7	4.3	3.5	
	$\Delta$	0	0	0	
12 B.J	<i>t</i>	20 ( <i>f</i> )	40	61	
Experimental	<i>P</i>	8.2	4.4	2.7	
Calculated	<i>P</i>	8.2	4.5	2.7	
	$\Delta$	0	+ 0.1	0	
13 J.P	<i>t</i>	21 ( <i>f</i> )	41	61	81
Experimental	<i>P</i>	7.2	4.8	3.4	2.6
Calculated	<i>P</i>	7.2	4.9	3.5	2.6
	$\Delta$	0	+ 0.1	+ 0.1	0
14 B.D	<i>t</i>	23 ( <i>f</i> )	42	61	
Experimental	<i>P</i>	4.8	3.0	2.1	
Calculated	<i>P</i>	4.8	3.1	2.1	
	$\Delta$	0	+ 0.1	0	

*t*=minutes following injection

Experimental and calculated *P*=mg diodone iodone/100 ml plasma water

$\Delta$  indicates the difference between the experimental and calculated *P*

(*f*) indicates the time on the plasma diodone iodine time curve where the formula is fitted

TABLE IX—COMPARISON OF VALUES OF THE PERCENTAGE CONCENTRATION OF DIODONE IODINE IN PLASMA WATER DERIVED FROM THE BEST CURVE DRAWN THROUGH EXPERIMENTALLY DETERMINED POINTS WITH THOSE CALCULATED ACCORDING TO THE FORMULA

$$P = \frac{K V_D \frac{-(C' + m)}{m} - T'm_D}{C' + m}$$

THE AMOUNT OF DIODONE INJECTED IN ALL CASES WAS SUFFICIENT TO MAINTAIN TUBULAR SATURATION FOR THE DURATION OF THE EXPERIMENT

Subject									
1 M.R	<i>t</i>	10	20 ( <i>f</i> )	40	60	80	100	120	
Experimental	<i>P</i>	95.7	85.1	67.0	52.7	41.8	33.0	26.1	
Calculated	<i>P</i>	97.9	85.1	66.0	51.7	41.1	32.8	26.1	
	$\Delta$	+ 2.2	0	- 1.0	- 1.0	- 0.7	- 0.2	0	
2 J.S	<i>t</i>	20	32 ( <i>f</i> )	55	80	96			
Experimental	<i>P</i>	64.5	50.0	34.8	25.2	21.3			
Calculated	<i>P</i>	59.7	50.0	36.8	26.1	21.3			
	$\Delta$	- 4.8	0	+ 2.0	+ 0.9	0			

TABLE IX—Continued

5	W.H	<i>t</i>	10	24 ( <i>f</i> )	44	63	84	105	125
	Experimental	<i>P</i>		97.8	70.0	55.0	43.4	35.5	28.5
	Calculated	<i>P</i>	119.2	97.8	74.3	59.1	45.7	36.1	28.5
		$\Delta$		0	+ 3.4	+ 3.2	+ 2.2	+ 0.6	0
6	R.D	<i>t</i>	17	27 ( <i>f</i> )	45	65	86	105	125
	Experimental	<i>P</i>	76.0	62.2	49.1	39.4	31.9	26.6	21.8
	Calculated	<i>P</i>	70.0	62.2	51.2	41.6	33.1	27.0	21.8
		$\Delta$	- 6.0	0	+ 2.1	+ 2.2	+ 1.2	+ 0.4	0
7	M.M	<i>t</i>	10	28 ( <i>f</i> )	49	68	88	108	
	Experimental	<i>P</i>	89.1	56.4	39.2	30.3	23.4	18.6	
	Calculated	<i>P</i>	73.1	56.4	41.6	32.2	24.5	18.6	
		$\Delta$	- 16.0	0	+ 2.4	+ 1.9	+ 1.1	0	
8	C.B	<i>t</i>	15	25 ( <i>f</i> )	44	64	84	104	
	Experimental	<i>P</i>	92.0	76.1	57.4	43.6	34.0	27.7	
	Calculated	<i>P</i>	87.6	76.1	57.6	44.6	35.0	27.7	
		$\Delta$	- 4.4	0	+ 0.2	+ 1.0	+ 1.0	0	
9	G.M	<i>t</i>	20	30 ( <i>f</i> )	49	69	89	109	
	Experimental	<i>P</i>	97.8	79.0	60.5	47.8	40.5	34.2	
	Calculated	<i>P</i>	89.5	79.0	63.4	51.4	41.6	34.2	
		$\Delta$	- 9.3	0	+ 2.9	+ 3.6	+ 1.1	0	
10	M.L	<i>t</i>	15	25 ( <i>f</i> )	46	67	86	106	128
	Experimental	<i>P</i>	107.7	91.4	68.8	55.9	47.3	40.3	34.4
	Calculated	<i>P</i>	100.5	91.4	73.4	60.6	50.3	41.9	34.4
		$\Delta$	- 7.2	0	+ 4.6	+ 4.7	+ 3.0	+ 1.6	0
11	J.D	<i>t</i>	10	22 ( <i>f</i> )	32	43	87		
	Experimental	<i>P</i>	39.8	33.5	30.3	27.9	20.0		
	Calculated	<i>P</i>	37.0	33.5	30.7	28.2	20.0		
		$\Delta$	- 2.8	0	+ 0.4	+ 0.3	0		
12	B.J	<i>t</i>		29 ( <i>f</i> )	49	69	88	109	129
	Experimental	<i>P</i>		76.8	58.9	48.4	41.1	34.7	29.4
	Calculated	<i>P</i>		76.8	63.2	52.4	43.7	35.8	29.4
		$\Delta$		0	+ 4.3	+ 4.0	+ 2.6	+ 1.1	0
13	J.P	<i>t</i>	19	29 ( <i>f</i> )	51	71	92	114	
	Experimental	<i>P</i>	106.0	94.6	76.3	64.5	54.8	48.4	
	Calculated	<i>P</i>	105.7	94.6	78.0	67.3	56.8	48.4	
		$\Delta$	- 0.3	0	+ 2.3	+ 2.8	+ 2.0	0	
14	B.D	<i>t</i>	16	26 ( <i>f</i> )	46	65	86	106	126
	Experimental	<i>P</i>	107.0	86.8	66.3	57.0	49.2	41.0	40.0
	Calculated	<i>P</i>	94.2	86.8	71.0	61.3	52.9	45.9	40.0
		$\Delta$	- 12.8	0	+ 4.7	+ 4.3	+ 3.7	+ 1.9	0

*t* = minutes following injectionExperimental and calculated *P* = mg diiodone iodine/100 ml plasma water $\Delta$  indicates the difference between the experimental and calculated *P*(*f*) indicates the time on the plasma diiodone iodine time curve where the formula

is fitted

## DISCUSSION

The authors have recently described a method [1949] by which an accurate estimation of the renal clearance of inulin could be obtained from the falling plasma inulin-time curve following a single injection and involving the collection of urine approximately 30 and 120 minutes following injection. The advantages of the method consist of a very large degree of technical simplification, and abolition of the need to approximate, by direct experiment, the instantaneous rate of inulin excretion in the urine. The applicability of the principles of the method to the determination of the renal clearance of diodone and the maximal tubular excretory power for diodone has now been demonstrated. By taking cognisance of the volumes of distribution at two times following the injection of diodone and assuming a linear relationship between the volumes of distribution and time, results for both determinations have been obtained which closely correspond to the average values of determinations for short clearance periods, following the intravenous injection of diodone. The degree of error introduced by the one assumption which is made may be expressed in terms of the ability to predict the plasma levels of diodone, employing formulæ (3) and (8), and it has been shown that the prediction is accurate within the limits of error of the chemical determination of diodone iodine in the plasma. The assumption is also justified in the sense that the linear relationship imposed is merely the graphical expression of the average rate of change of  $V_D$  between the limits of time selected.

For the purpose of comparing the two methods, urine was collected at intervals of approximately 20 minutes from all subjects, but this procedure is unnecessary for the practical application of the formulæ. To determine either the  $Tm_D$  or the renal clearance of inulin, the interval between the urine collections can be extended to about 90 minutes so that spontaneous passage of urine may be allowed. As a result of this, errors of collection are reduced to insignificance in the vast majority of subjects. The stated need to maintain the plasma levels of diodone, for measurements of  $C_D$ , between the limits of 0.5 and 5 mg/100 ml plasma [Smith *et al.*, 1938], tends to limit the interval between the times of urine collection when the diodone is injected intravenously. In the experiments described, this period has been approximately 40 minutes. Whether this limitation is really necessary depends upon, *inter alia*, a reconsideration of the influence of the plasma level of diodone upon its renal clearance, especially between 5 and 10 mg diodone iodine/100 ml.

Two values of the volume of distribution of the injected substance having been determined, a knowledge of the plasma levels of the substance at these times completes the experimental data required for calculating the value of  $C_D'$ . In the determination of values for  $Tm_D$ , estimations of the rate of glomerular filtration by measurements of

inulin clearance are also necessary. In the subjects studied here, measurements of inulin clearance have been made simultaneously with measurements of the renal clearance of diodone, one injection being given immediately after the other. It is possible to give the inulin injection in association with diodone in amounts sufficient to measure  $Tm_D$ , though it is clear that measurements of  $C_D$  and  $Tm_D$  may not be made simultaneously. It is convenient in routine estimations to combine measurements of inulin clearance and tubular excretory capacity, and to perform an independent estimation of the renal clearance of diodone either immediately prior to this procedure or on a subsequent day.

In the estimation of the clearance of diodone and inulin and the tubular excretory capacity for diodone, the values for plasma concentration have been obtained by sampling venous blood. This practice necessarily introduces some error, whatever technique is used, since the kidney is supplied with arterial blood. The error, the magnitude of which depends on the arterio-venous difference, is likely to be larger in all single injection methods than in those employing continuous infusion, because, in the former, this difference is determined by the ratio between the renal clearance and the cardiac output [Brun, Hilden and Raaschou, 1949]. On this basis, it appears that the arterio-venous difference is small enough to be ignored in estimations of the renal clearance of inulin and the maximum tubular excretory capacity for diodone following single injections of these substances, since in normal people the total amounts of inulin or diodone cleared in one circulation are contained in volumes of blood approximately 4 and 8 per cent of the cardiac output respectively. In renal disease, where there is a disproportionate reduction in renal blood-flow as compared with cardiac output, the arterio-venous difference will tend to be less.

In one adult subject (inulin clearance 91 ml plasma/minute,  $Tm_D$  41.7 mg diodone iodine/minute) in whom estimations of plasma inulin and diodone were made on samples of arterial and venous blood eight times following a single intravenous injection of inulin and diodone, the authors found the mean arterio-venous difference of inulin to be 3 per cent and of diodone to be 8 per cent. The error in inulin clearance in using venous blood is represented by the mean arterio-venous difference, i.e. 3 per cent in this subject. In the case of  $Tm_D$ , however, the error introduced by the use of venous blood instead of arterial blood is less than that represented by the arterio-venous difference, i.e. 8 per cent in this subject. This follows from the fact that the term  $C_{in}P_D$  in the basic  $Tm_D$  formula includes an underestimate of  $C_{in}$  (3 per cent) and an overestimate of  $P_D$  (8 per cent). When, however, a single intravenous injection of diodone is given for the purpose of establishing low plasma levels of diodone suitable for the estimation of the effective renal plasma-flow, and venous blood is analysed, the error in the clear-

ance of diodone is likely to be of the order of 20 per cent in normal persons, since the renal blood-flow approximates to one-fifth of the cardiac output. For clinical purposes this is no real disadvantage, but a more precise knowledge of the effective renal plasma-flow demands analysis of arterial blood samples. Two such samples having been obtained, the method described by the authors can be applied.

The estimation of the renal clearance of diodone at low plasma levels suffers from the further disadvantage that the plasma level must be kept within the range of 0.5 to 5 mg/100 ml [Smith *et al*, 1938]. The maintenance of constancy in the plasma level is a matter of great technical difficulty when infusion is employed, and, with the authors' technique, it tends to limit the interval between the times of urine collection to approximately 40 minutes. In addition, the rapidity of the fall of the plasma-diodone concentration after a single injection exaggerates errors due to the uncertainty of the duration of the interval between the production of urine in the renal tubules and its arrival in the bladder. It is clear that a closer definition of this time interval and of the arterio-venous difference following the intravenous injection of diodone is necessary for estimations of the renal clearance of diodone, employing the single injection method, to acquire greater precision than that required in clinical work.

The physiological and clinical value of inulin and diodone clearances is largely dependent upon the validity of the claims that these clearances represent the glomerular filtration rate and the renal plasma-flow respectively. It is proposed to investigate these claims in subsequent publications. It is relevant to the present discussion, however, to suggest that the adoption of other compounds for the evaluation of these renal processes, such as the substitution of para-acetyl-amino hippuric acid for diodone [Newman, Kattus, Gerrian, Genest, Calkins and Murphy, 1949], does not affect the validity of the theoretical principles applied by the authors to inulin and diodone. The same principles are equally applicable to any compounds which may replace them, and which will, *ex hypothesi*, approximate even more closely to the ideal behaviour.

## SUMMARY

1 The principles devised by the authors [1949] for the determination of the renal clearance of inulin have been applied to the determination of the renal clearance of diodone and the tubular excretory capacity for diodone.

2 Following the intravenous injection of diodone in amounts suitable for the measurement of both renal clearance and tubular excretory capacity, the volume of distribution of diodone continues to increase for the duration of the experiments. It is concluded that

equilibration of diodone between the plasma and the extravascular component of the volume of distribution does not occur under the conditions of the experiment

3 Formulæ are derived for the renal clearance of diodone and the maximal tubular excretory capacity for diodone incorporating functions which are demanded by the failure of the establishment of equilibration

4 The formula for the renal clearance of diodone is applied to fourteen subjects possessing widely different clearance values, and the formula for the maximal tubular excretory capacity for diodone is applied to twelve of these subjects. The values calculated by the formulæ agree closely in every case with the average values experimentally determined, following intravenous injections of diodone

5 The calculative errors introduced into the formulæ by the simplifying assumption that the volume of distribution of diodone is linearly related to time, between defined limits of time, are shown to be of similar magnitude to the limits of the error in the chemical determination of diodone in the plasma

6 The formulæ can be applied without the need for accurate urine collection over short clearance periods. They necessitate the passage of urine approximately 25 and 120 minutes (for the maximal tubular excretory capacity for diodone) and 20 and 60 minutes (for the renal clearance of diodone) after the injection of known amounts of diodone, and a minimum of two blood samples for each determination taken at the time of urine passage

7 The errors introduced in the determinations of inulin and diodone clearance and the maximal tubular excretory capacity for diodone by sampling venous blood are discussed briefly

8 The results of a further five estimations of inulin clearance employing the authors' method (*loc cit*), and their comparison with average values experimentally determined, are also included

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SOME OBSERVATIONS ON THE BRONCHOCONSTRICTOR  
EFFECTS OF TUBOCURARINE By M MAHFOUZ From  
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THE occurrence of bronchospasm was reported by West [1936] to follow the injection of curarine in some cases of Parkinsonian rigidity. It was believed that this effect was due to the presence of an impurity contaminating the sample used. Several clinical reports continued to mention the occurrence of an undesirable and dangerous side reaction with a sudden onset, following upon the administration of curare preparations [Cullen, 1944, Harvey and Masland, 1941]. The reaction was described as respiratory difficulty accompanied by cyanosis. Increased resistance to inflation of the lungs by manual compression on the breathing-bag has been reported by Whitacre and Fisher [1945], and by Holaday [1946], to occur in some anaesthetized patients given intocostin. This respiratory spasm was observed to be relieved by another injection of the drug. In reports on animal experiments, a reaction to curare administration, similar to that occurring in man, has been described. Cole [1946] reported the development of cyanosis in dogs following the administration of intocostin, despite vigorous artificial respiration through an endotracheal tube, and he attributed this condition to bronchospasm.

Since Alam, Anrep, Barsoum, Talaat and Weininger [1939] demonstrated the release of histamine as a result of curarine injection, evidence has been accumulating of the occurrence of histamine reactions following the injection of the most purified preparations, intocostin and tubocurarine. Comroe and Dripps [1946] showed that certain vascular effects of intocostin in man, *i.e.* the wheal-and-flare response, were similar to histamine reactions. Grob, Harvey and Lihenthal [1947], reported similar reactions with *d*-tubocurarine chloride. Feldberg and Holmes [1941] reported that the injection of curarine, like that of histamine, caused gastric secretion of free HCl. Landmesser [1947] has recently shown that bronchoconstriction occurred in spinal dogs as a result of the injection of *d*-tubocurarine chloride, and that that was due to the histamine liberated. He used the Drinker-Murphy infant resuscitator adapted to record bronchial calibre by the plethysmographic method of Jackson.

Results similar to those of Landmesser were obtained here using  
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spinal guinea-pigs with open chests. These animals were chosen as they have been shown to be most susceptible to the action of tubocurarine, and if the bronchoconstrictor properties of this drug are due to the liberation of histamine, the effects of this substance may be more easily reflected in the guinea-pig's bronchi.

## METHODS

### *A. Recording of Bronchoconstriction*

' Decerebrate guinea-pigs of both sexes, weighing 500–650 g, were used. The animal was anaesthetized with ethyl ether and a tracheal cannula, with a side tube, introduced into the trachea, and the anaesthesia continued through a Wolff's bottle, until the brain was destroyed. Then artificial respiration was started by means of a pump attached to the trachea. The chest cavity was opened by severing the junction between the xyphoid process and the body of the sternum, then cutting the latter upwards in the middle line for about 2–2½ inches from its lower margin. The two edges of the chest wall were fixed moderately apart by rigid clamps. The opening thus made in the chest was well covered by warm, moist gauze. The side tube in the tracheal cannula was then connected by pressure tubing to a recording tambour, moving a lever which amplified its movements about 20 times and recorded them on a smoked drum. A sensitive tambour of about 4½ inches diameter covered with thin rubber was used. The air delivered by the pump is distributed between the bronchi and the tambour, and the state of balance recorded by the lever on the drum. Constriction in the bronchial calibre results in deflection of incoming air to the tambour, causing an increase in the amplitude of the record. Better records were obtained with the chest opened than when it was closed.

The drugs were injected intravenously through a cannula connected to a superficial neck vein. Tubocurarine was used in doses of 0.03 mg in 1 c.c. saline per kg body-weight, histamine as 0.01 mg of the base in 1 c.c. saline per kg body-weight, unless otherwise mentioned. For protection with neoantergan, this drug was given in doses of 1 mg per kg subcutaneously ½–1 hour before the operation, and for the treatment of an attack it was given as 0.1–0.2 mg in 1 c.c. saline per kg body-weight, i.v. All i.v. injections were given rapidly (i.e. in 2–3 sec) unless otherwise indicated. The slow intravenous infusion of tubocurarine was controlled by a capillary glass tube in the top end of a full burette whose outflow end was connected to the venous cannula. The drug was infused at the rate shown by the air-bubbles leaving the lower end of the capillary tube, and this was adjusted so that the appropriate dose would flow over a period of 1 minute or slightly more.

### B *Inhalation of Tubocurarine*

For this purpose a Collison spray atomiser was used with a small container for the drug

The cloud was produced by passing a continuous stream of compressed air into the aqueous solution in the container. The air was delivered from a compressed air cylinder at a pressure of 20 lb per square inch and maintained constant by an adjusting screw. The cloud was then taken by a curved glass tube to just below the centre of a wire-mesh platform fitted horizontally inside a large glass desiccator. The upper part of the desiccator was divided by a vertical wire-mesh screen into two compartments of equal size each holding one animal. The cloud escaped by a tube fitted into a hole drilled in the centre of the desiccator dome. The output of the atomizer was about 10 litres of air and 0.7 c.c. of watery solution per minute. Tubocurarine was used as 3 per cent watery solution. Two guinea-pigs of the same sex and weight were used at a time, one in each compartment of the animal chamber. One of the animals usually received a subcutaneous injection of 1 mg per kg body-weight neoantergan  $\frac{1}{2}$ -1 hour before starting the spray. The duration of spraying was 15-20 minutes.

## RESULTS

### A *Intravenous Injection*

The intravenous injection of tubocurarine in the unprotected animal caused bronchoconstriction, reaching its maximum in about half a minute and passing off gradually in about 4 minutes. A second intravenous dose of the drug about 6 minutes after the first was then without any effect.

The subsequent injection of histamine, however, produced a bronchospasm, reaching its maximum also in about half a minute, but lasting longer than the response to tubocurarine. Larger initial doses of tubocurarine did not appreciably increase the degree of constriction. The refractoriness of the animal to a second dose of tubocurarine, the effects of the first dose having passed off, was still there, even when this second dose was greater than the first and given after a long interval. Fig 1 is an example of such effects produced in a guinea-pig weighing 550 g. Of 18 guinea-pigs, however, 7 failed to respond to the first rapid injection of tubocurarine, though not to histamine.

In experiments on 7 further guinea-pigs, the animals were protected  $\frac{1}{2}$ -1 hour before the operation by the subcutaneous injection of neoantergan 1 mg per kg body-weight. In these cases the rapid injection of tubocurarine did not precipitate an attack of bronchoconstriction. Since bronchoconstriction did not invariably occur in unprotected

guinea-pigs, a single observation of this kind would mean little. Out of 18 unprotected guinea-pigs 11 responded, and out of 7 protected guinea-pigs none responded. Calculation supports the view that this difference of result is significant. The effect of neoantergan is illustrated in fig 2. In this case tubocurarine was rapidly injected fully 60 minutes after the animal had received its subcutaneous protection. No constriction resulted. When the histamine injection followed half an hour later a very slight transient effect was seen.

When the dose of tubocurarine was injected slowly in the unprotected animal, over a period of one minute or more, no bronchoconstriction occurred. After the slow intravenous infusion, a dose of the drug injected rapidly produced the usual bronchoconstrictor effects. Then when a second rapid injection of the drug was given, after the effects of the first dose had passed off, the animal showed again the usual refractoriness, but was not refractory to the injection of histamine that followed. Fig 3 is an example of such an experiment.

In another series of experiments it was decided not to give any degree of protection to the animals beforehand, but to develop in them an attack of tubocurarine bronchoconstriction, and then see how far such an attack could be modified by the administration of neoantergan at various stages of its development. Fig 4 shows the production of such an attack by the rapid intravenous administration of tubocurarine. When the attack was at its peak, the rapid injection of neoantergan apparently relieved it and restored the bronchial calibre to some degree. About one hour later the effects of the antihistamine seemed to have weakened, and when a large dose of histamine was given, this caused a bronchoconstriction, which was again effectively treated at its peak by intravenous neoantergan. The first part of this curve shows that the slow injection of tubocurarine had no effect on the bronchi.

When an attack was left to pass its peak and get fully developed, the neoantergan treatment seemed to relieve it to some degree. Fig 5 is an example of such responses. If an attack was dealt with early enough by the prompt administration of neoantergan, its full development was apparently suppressed.

### B *Inhalation*

In the inhalation experiments, guinea-pigs were exposed to an aerosol of aqueous solution of tubocurarine with and without preliminary protection by neoantergan. The inhalation was meant to obviate, at least during the early part of the experiment, the general action of the drug, in order to get the effect of its direct application upon the bronchial system of the living animal. No signs of bronchoconstriction were observed in any of the animals upon such an inhalation, whether they had or had not been protected beforehand.

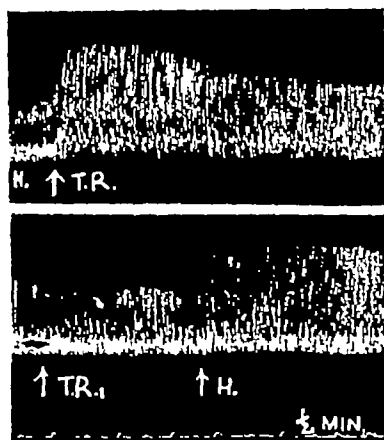


FIG 1

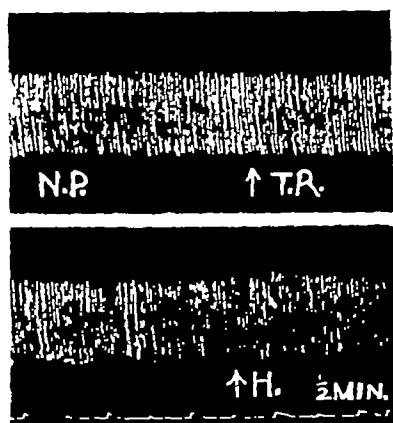


FIG 2

FIG 1—Bronchoconstriction in guinea pig (550 g) following the rapid intravenous injection of tubocurarine, with refractoriness of the animal to a second injection

T R tubocurarine injected rapidly (10.5  $\mu$ g) T R<sub>1</sub> tubocurarine (86  $\mu$ g) similarly injected 90 minutes later H histamine (5.5  $\mu$ g)

FIG 2—Prevention of tubocurarine bronchoconstriction in guinea pig (520 g) by previous protection with neoantergan

N P normal record of an animal protected with neoantergan (1 mg/kg) subcutaneously 1 hour previously T R tubocurarine (60  $\mu$ g) injected rapidly H histamine (30  $\mu$ g)

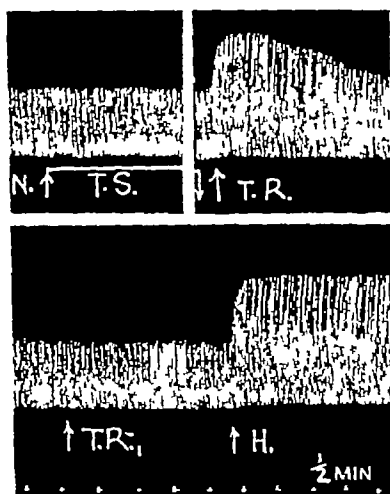


FIG 3—Prevention of tubocurarine bronchoconstriction in guinea pig (500 g) by the slow intravenous administration of the drug

N normal T S tubocurarine (225  $\mu$ g) injected slowly at a rate of 15  $\mu$ g/min T R tubocurarine (15  $\mu$ g) injected rapidly T R<sub>1</sub> same dose similarly injected 7 minutes later H histamine (5  $\mu$ g) Guinea pig (500 g)

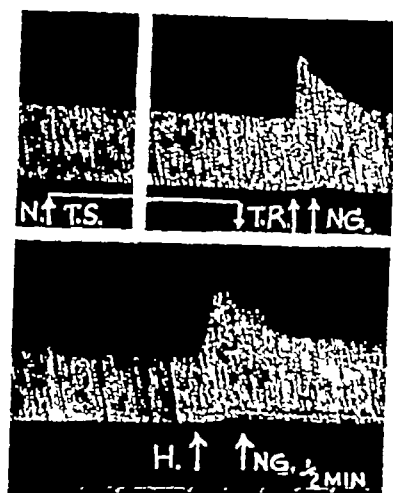


FIG 4—Interruption of an attack of bronchoconstriction (at its peak) caused by tubocurarine in the guinea pig (500 g) by the prompt intravenous administration of neoantergan

T S tubocurarine (150  $\mu$ g) infused slowly over a period of 10 minutes I R tubocurarine (15  $\mu$ g) injected rapidly N G neoantergan 0.1 mg H histamine (70  $\mu$ g) given 1 hour after T R

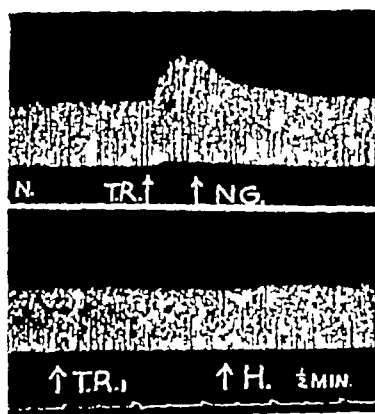


FIG 5—Interruption of an attack of bronchoconstriction (fully developed) caused by tubocurarine in the guinea pig (600 g) by the intravenous administration of neoantergan

T R tubocurarine (18  $\mu$ g) injected rapidly N G neoantergan 0.12 mg  
T.R. tubocurarine (36  $\mu$ g) injected rapidly H histamine (10  $\mu$ g) given half an hour after T R

with neoantergan At the end of the period of spray, however, the animals exhibited some signs of the general action of the drug in the form of a moderate degree of paralysis, from which they recovered in about 7-10 minutes

When these guinea-pigs were exposed the following day to an aerosol of an aqueous solution of 3 per cent histamine base, after giving a fresh protective injection to the control, the unprotected guinea-pig showed the usual signs of severe histamine bronchospasm, which sometimes resulted in loss of the animal In the control animals protection was complete

### *C Experiments on the Conscious Human Subject*

I Here experiments were conducted on a 75 kg healthy medical man (self), not undergoing operation and with no concurrent pathological history The drug, tubarine "B W," was injected in a small single dose of 7.5 mg slowly intravenously over a period of two minutes One minute after the beginning of the injection, after about 3.5 mg had been injected, there were definite myasthenic symptoms and descending paralysis involving the eyelids, face, neck muscles and the extremities The movements of the eyes became sluggish and the gaze tended to be fixed There was also some heaviness of the tongue, with difficulty of speech

Although these symptoms were increasing in severity and towards the end of the injection were actually alarming, no respiratory difficulty was felt

II On a later occasion 15 mg of tubarine "B W" was injected into the same subject rapidly intravenously in a few seconds A means of communication had been established beforehand between the subject and the operators, since paralysis of the tongue muscles was almost certainly expected For a period of not less than half a minute after the injection muscular power was almost full and no symptoms were at all experienced, then the subject was suddenly caught by a severe asthmatic attack At this stage muscular power was apparently still present, because the subject was able to move his arm, pointing with his palm to the face asking for the oxygen mask The mask was put on, and artificial respiration conducted with oxygen under positive pressure

Despite this artificial aid to the respiration, the respiratory embarrassment maintained its severity for about three minutes, during which the subject was thrown into occasional convulsions, probably anoxæmic Shortly afterwards he was lying flaccid, with full ptosis and muscular paralysis The tongue did not fall to the back of the throat, and the upper airways were not plugged with mucous

Consciousness was not lost and the memory was not clouded There

were no evident changes in the sensations. The colour of the skin was normal or slightly pinkish, and there was profuse sweating of the brow. The pulse was accelerated (from 78 to 90 per minute), and the blood-pressure slightly raised (from 145/90 to 165/100 mm Hg) during the fifteen minutes following the injection, it then gradually came down to the original level.

III. The purpose of the following experiment, done on an asthmatic patient, was (a) to find out whether the histamine, known to be liberated as a result of the intravenous injection of tubocurarine, would precipitate an attack of asthma in a presumably sensitive individual, (b) to test if there would be any increase in the histamine content of the urine following upon such an injection.

The patient, a male 45 years old, has been suffering from asthma since 1942. His attacks are readily removed by adrenaline. Tubarine "B W" was injected very slowly intravenously, 10 mg being given over a period of three minutes. Although there was definite ptosis, difficulty of speech, with sweating and flushing of the cheeks, no subjective attack of asthma was complained of. No increase in the histamine content of the urine was detectable in the first three hourly specimens, or in the 24-hours' pooled sample.

### DISCUSSION

Probably the vital system whose responses provide the most important contraindication to the use of tubocurarine is the respiratory system. Therefore, before administering the drug, one must be convinced of his ability to care for the respiratory depression that may ensue. Artificial respiration applied by the Schaefer or similar method may completely fail to resuscitate, and thus means of artificial respiration under positive pressure should always be available. These methods, however, may prove inadequate if the bronchial calibre is seriously interfered with.

Reference has already been made to various clinical accounts of the occurrence of asthma. Experiment II, described above, confirmed the fact that this may occur in man, and suggested that its occurrence depended on the speed of injection. The experiments on guinea pigs showed clearly that bronchoconstriction could be produced by comparatively small doses injected rapidly in conditions where very much larger doses, given slowly, had no apparent effect on the bronchi. The antagonistic effect of neoantergan suggests that this phenomenon is due to the release of histamine, and this theory is also supported by the fact that it is already known that curare does release histamine. The failure of a second injection of tubocurarine to cause bronchoconstriction can be explained on the theory that all the histamine available for immediate release is released by the first injection. The



failure of aerosols of tubocurarine to cause asthma confirms the view held by Alam *et al* [1939] that the lungs themselves are not the main source of the histamine which is liberated

These experiments suggest the practical conclusion that the occurrence of asthma following injections of tubocurarine is likely to be less frequent when the injection is given slowly than when it is given rapidly. If neoantergan were always administered with curare it is very unlikely that asthma would ever occur, when it does occur, the injection of neoantergan is likely to stop it

### SUMMARY

A rapid intravenous injection of tubocurarine usually causes bronchoconstriction in guinea-pigs. A second injection has no such effect. The effect may be prevented or interrupted by neoantergan, and is probably due to the release of histamine. The best way to avoid this effect in the clinical use of tubocurarine is to give the injection slowly. Slow injections of large doses in guinea-pigs had no effect on the bronchi, although a dose 10-15 times smaller caused bronchoconstriction when given rapidly.

### ACKNOWLEDGMENTS

It is a pleasure to acknowledge the encouragement and guidance of Professor J. H. Gaddum, Dr Ranyard West and Dr M. Vogt. My thanks extend to Dr J. Gillies and Dr H. W. Griffith of the Anaesthetic Department for their help in the experiments on the conscious subjects, to Dr H. Adam of this Department for estimating the histamine content of the urine of the asthmatic patient, to Dr J. W. Trevan, Director of the Wellcome Physiological Research Laboratories, for supplying the sample of tubocurarine used in the animal experiments, and to the Moray Research Fund of Edinburgh University for defraying some of the expenses of this work.

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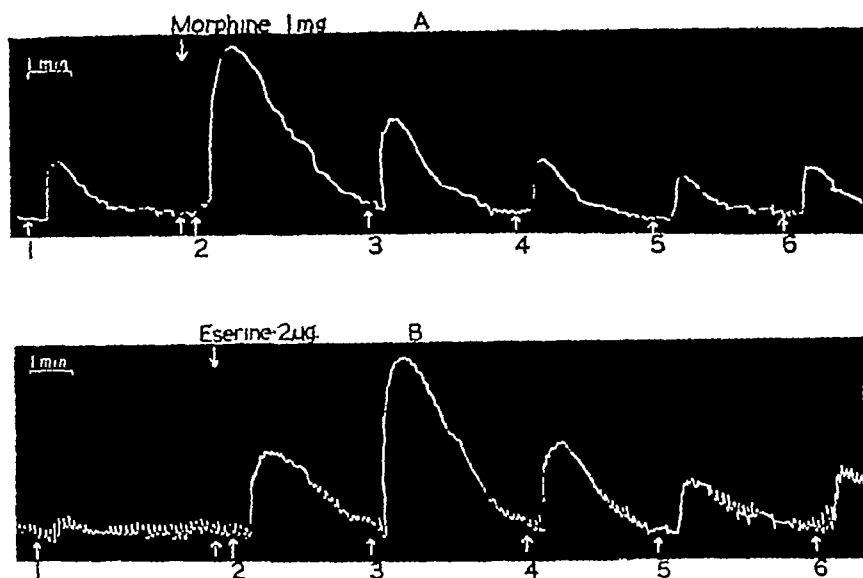


FIG 1—Contractions of nictitating membrane during perfusion of denervated superior cervical ganglion (right) in response to single injections of acetylcholine ( $0.2 \mu\text{g}$ )

(A) Potentiating action of morphine ( $1 \text{ mg}$ )

(B) Similar effect of eserine ( $2 \mu\text{g}$ ) (Right cervical sympathetic nerve sectioned 12 days before experiment)

Between the end of (A) and beginning of (B) there was an interval of 31 minutes

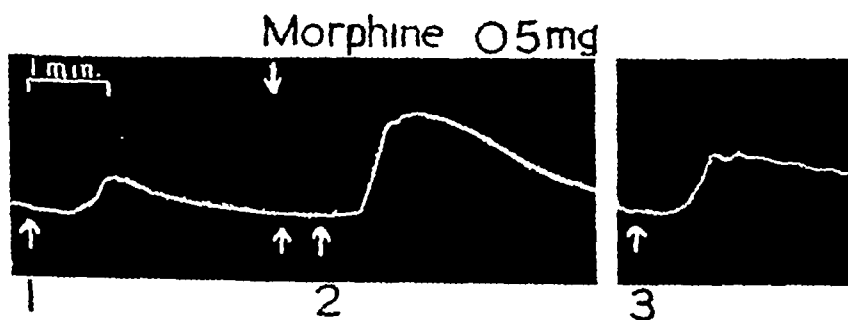


FIG 2—Contractions of nictitating membrane during perfusion of denervated superior cervical ganglion (right) in response to  $0.8 \text{ mg}$  KCl. Potentiation of responses by morphine ( $0.5 \text{ mg}$ ) (Right cervical sympathetic nerve cut 7 days before experiment) Between the 2nd and 3rd injections there was an interval of 7 minutes



THE EFFECT OF CERTAIN ANALGESIC DRUGS ON SYNAPTIC TRANSMISSION AS OBSERVED IN THE PERFUSED SUPERIOR CERVICAL GANGLION OF THE CAT By CATHERINE O HEBB and HERIBERT KONZETT From the Department of Physiology, University of Edinburgh

(Received for publication 5th February 1949)

PETHIDINE and amidone, two new synthetic drugs, share with morphine the properties of relieving pain [Schaumann, 1940, Batterman and Himmelsbach, 1943, Scott and Chen, 1946] and of producing addiction [Himmelsbach, 1942, Wikler and Frank, 1947], while they differ from morphine in certain other pharmacological actions [Yonkman, Noth and Hecht, 1944]

As yet no systematic study of their effects on the autonomic nervous system has been made, although this might well be of interest in view of the fact that, like morphine, both pethidine and amidone inhibit brain and serum cholinesterases [Brindley, 1944, Eadie, Bernheim and Fitzgerald, 1948, Greig and Howell, 1948] If the anti-cholinesterase activity of these drugs is an important feature of their action, one might expect that they could modify the conditions for transmission of nervous impulses through autonomic ganglia To test this possibility we have carried out experiments on the perfused superior cervical ganglion [Kibjakow, 1933]

#### METHODS

Cats, 2.3-3.4 kg, were anaesthetised with chloralose (0.08-0.1 g/kg intravenously) following induction with ether In the preparation and perfusion of the ganglion we used the method introduced by Kibjakow [1933], as later modified by Feldberg and Gaddum [1934] A water-jacket was used instead of a heating-coil for warming the perfusion fluid (modified Locke solution with low K, see Feldberg and Gaddum, 1934) The fluid was twice filtered, first through a No. 4 and then through a No. 5 sintered glass filter (filters as supplied by Messrs Griffin & Tatlock) During perfusion it was aerated with pure oxygen

For nerve stimulation a Ritchie-Sneath square wave stimulator was used A good response was usually obtained when the strength of stimulus was 0.5 volt applied for 5 seconds or less (pulse duration of 1 msec, frequency of 5 per sec)

Injections were made by means of a long hypodermic needle (capacity, 0.02 c.c.), which was inserted through a side arm of the cannula and pushed forward so that its tip lay close to the opening into the artery. The drugs administered (eserine sulphate, acetylcholine, pethidine HCl, *dl*- and *l*-amidone) were made up in filtered modified Locke solution.

In some experiments the superior cervical ganglion was denervated by the removal aseptically of a short length of the cervical sympathetic 6-12 days before experiment.

Contractions of the nictitating membrane were recorded isotomically by means of a lever equipped with a frontal writing-point.

## RESULTS

*Morphine*—Tests were made to determine whether morphine given in single injections (doses from  $2.5 \times 10^{-4}$  to 2 mg) or perfused continuously through the ganglion (0.5-50  $\mu$ g per c.c. of perfusion fluid) would modify the responses of the nictitating membrane to pre-ganglionic nerve stimulation, injection of acetylcholine (1-20  $\mu$ g) or KCl (0.3-1.0 mg). Morphine given in this way produced no measurable effects itself on the muscle. It also appeared that the drug caused no significant change in the excitability of the normally innervated ganglion to the stimuli mentioned. Some experiments suggested that given in single injections it had a brief and very slight depressant action, but this could not be confirmed in other experiments in which there was either no evidence of such an effect, or some indication that the excitability of the ganglion was increased by morphine. Continuous perfusion of the ganglion with morphine (1  $\mu$ g/c.c.) for long periods (e.g. 1 hour) could also be carried on without evidence of a change in the responses either to nervous or chemical (acetylcholine) stimulation.

In contrast to normally innervated ganglia, those which had been denervated showed a marked increase in excitability after single injections of morphine in doses varying from 0.1 to 2.0 mg. With such doses the response of the nictitating membrane to acetylcholine given some 20-40 seconds later was much larger than the control responses (fig. 1, A). The effect generally persisted for a few minutes only, and was sometimes succeeded by a period of diminished excitability. In the experiment illustrated in fig. 1, A, the degree of potentiation of the action of acetylcholine produced by 1 mg of morphine was found to be equivalent to that produced by 2  $\mu$ g of eserine (fig. 1, B), although the effect of morphine was of shorter duration. The responses of denervated ganglia to KCl were similarly potentiated by morphine (fig. 2).

When the threshold dose of acetylcholine was high responses to this substance could only be potentiated by relatively large doses of morphine. Thus in experiments in which 10-20  $\mu$ g of acetylcholine were used as

test doses, the least effective dose of morphine was of the order of 1 mg, whereas in other experiments with greater sensitivity to acetylcholine as little as 100  $\mu$ g of morphine had a potentiating action

When given during perfusion of the ganglion with eserine (1 200,000), morphine did not produce any increase in the excitability over and above the increase in excitability due to eserine itself

*Pethidine*—Like morphine, pethidine, in doses up to 100  $\mu$ g, had no stimulant action on either normal or denervated ganglia, which could be detected by a response of the indicator muscle. Larger doses of pethidine were not tested, since, unlike morphine, it had a profoundly depressant effect on the perfused ganglion. In this its action was the same for both normal and denervated ganglia with or without perfusion with eserine (1 200,000). The reduction in responses of the nictitating membrane to acetylcholine, KCl and preganglionic nerve stimulation, due to injection of pethidine, is shown in fig 3. In this figure recovery of the response to nerve stimulation after the initial depression may also be seen. The degree and usually the duration of inhibition increased with the dosage of pethidine.

*Amidone*—Effects similar to those described for pethidine were obtained with *dl*- and *l*-amidone. These drugs differed from pethidine in the one respect that their depressant action was more powerful than that of pethidine. This difference was more marked in the case of *l*-amidone, which in doses as small as 1  $\mu$ g produced a significant depression of responses to acetylcholine, KCl or preganglionic stimulation, while the amounts of *dl*-amidone required to cause a comparable inhibition were between 5 and 10  $\mu$ g.

In certain experiments in which little or no recovery in the excitability of the ganglion had occurred after administration of a large dose of amidone, we found that the responses to nervous or chemical stimulation were restored by perfusion with eserine (1 200,000). Even during perfusion with eserine, however, the depressant effect of the drug could be demonstrated, although it was then necessary to use doses larger than those normally required (10  $\mu$ g of *l*-amidone, see fig 4). Similar results were obtained with amidone on denervated ganglia (fig 5).

## DISCUSSION

The depressant actions of pethidine and amidone here demonstrated are evidently related to effects exerted on the ganglionic cells or the post-ganglionic fibres, or both, since chemical and preganglionic nerve stimulation are equally affected, and analogous results are obtained after denervation. On the other hand, morphine not only has no significant depressant effect on normally innervated ganglia, but differs more sharply from amidone and pethidine in its ability to sensitize denervated ganglia to the stimulating actions of acetylcholine and KCl.

This sensitizing effect of morphine might be attributed to its anti-cholinesterase properties, but if so, it should also alter the excitability of the innervated ganglion cells, unless it is only effective when, as after denervation, the concentration of cholinesterases is reduced [Sawyer and Hollinshead, 1945]. Even so, it would be difficult on such an hypothesis to account for the potentiation of responses to KCl, since there is no reason to think that the stimulating action of KCl on denervated ganglia is mediated by acetylcholine. These considerations suggest, therefore, that the sensitizing effects of morphine on the denervated ganglion are independent of its anti-cholinesterase properties. Further, since both amidone and pethidine also possess such properties and are nevertheless strongly depressant, it is unlikely that the inhibition of cholinesterases plays any significant part in determining their effects.

The sympathetic ganglion cell has many properties in common with the neuromuscular end-plate, and it is of interest to compare the actions of amidone, pethidine and morphine on the ganglion with their actions on striated muscle. Torda and Wolff [1947] have shown that in high concentration (0.001 M) morphine increases the response of the frog rectus abdominis muscle to acetylcholine. This result we have been able to confirm. We have also found that pethidine in concentrations of  $10^{-3}$ – $10^{-4}$  M, and both *dl*- and *l*-amidone in concentrations of  $10^{-5}$  M, reversibly depress the motor responses of the muscle to acetylcholine (fig. 6). The effects of the three drugs on this muscle therefore correspond closely with their effects on the denervated sympathetic ganglion. It is again possible that the potentiating action of morphine depends upon an inhibition of the cholinesterases, but the depressant actions of amidone and pethidine are much stronger than any effects which might be derived from their anti-cholinesterase properties, *e.g.* by the accumulation of excess acetylcholine.

## SUMMARY

1. In the perfused superior cervical ganglion of the cat the administration of morphine does not affect the size of the contraction of the nictitating membrane produced by pre-ganglionic stimulation, injection of acetylcholine or of KCl when the pre-ganglionic fibres are normally functional, but after degenerative section of the cervical sympathetic nerve, morphine in large doses potentiates the contraction elicited by injections of acetylcholine and of KCl.

2. Pethidine and amidone strongly depress the responses of the membrane to pre-ganglionic stimulation and to injections of acetylcholine or KCl. Both drugs have a similar depressant action on responses to acetylcholine and KCl after denervation of the ganglia. In these actions, *l*-amidone is the most powerful and pethidine the least.



powerful, while *dl*-amidone is intermediate between the other two substances

3 Of the three analgesics tested, none have the power to excite a response of the nictitating membrane when injected into the ganglion

4 The effects of morphine, amidone and pethidine on the responses of the frog rectus abdominis muscle to acetylcholine are analogous to their effects on the responses of the denervated superior cervical ganglion to acetylcholine

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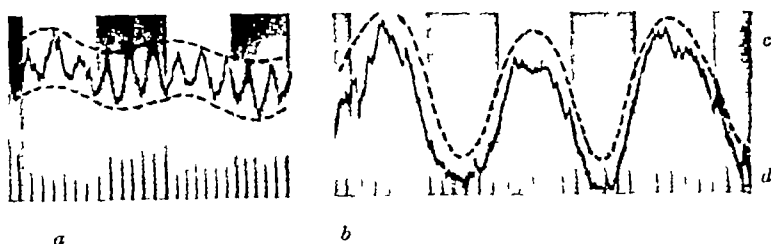


FIG 1—Specimen records (a) small blood flow (b) large blood flow. The curve whose amplitude is proportional to the blood flow is emphasized by the broken line deviations from it are of respiratory and cardiac origin (c) Signal marking periods of supra atmospheric pressure, (d) time in seconds

AN INSTRUMENT FOR THE STUDY OF VASCULAR FACTORS  
IN THE PHYSIOLOGY AND PATHOLOGY OF THE  
HUMAN MAMMARY GLAND By V R PICKLES, Physiology  
Department, King's College, Newcastle-upon-Tyne

(Received for publication 6th June 1949)

THE instrument to be described gives a continuous estimate of the blood-flow in the human mammary gland. No measurements of this quantity have hitherto been reported, since none of the standard methods of measuring blood-flow is readily applicable to the human breast. Only a few single measurements have been made on goats and cows [Jung, 1932, 1933, Graham *et al*, 1936, 1938, Shaw *et al*, 1942]. A detailed study of mammary blood-flow and its variations would be of considerable physiological and possibly of clinical interest.

The method to be described is a modification of the plethysmographic. It is applied to the whole intact organ, it involves no discomfort to the subject, and it is capable of registering fairly rapid fluctuations in the blood-flow. A preliminary account has already been published [Pickles, 1949].

The usual method of venous-occlusion plethysmography cannot be used, since it is anatomically impracticable to occlude all the veins of the breast. Instead, the air-pressure within the plethysmograph is reduced below atmospheric, causing a greater or less degree of stasis of venous blood and lymph in their respective vessels. The organ consequently swells and the plethysmograph air-pressure tends to return to atmospheric. It is in effect this rate of rise of pressure that is continuously recorded by the instrument, being almost directly proportional to the amplitude of the curve that the latter produces when the reduced pressure is applied rhythmically (fig 1—specimen records). The following considerations suggest that this amplitude is, under the appropriate conditions, very closely related to the mammary blood-flow.

Before the instrument itself is described, the volume changes that occur when the breast is subjected to subatmospheric external pressure must be considered in detail. The theoretical possibilities are summarized in fig 2.

- 1 Tissue may be sucked under the rim of the plethysmograph. Observation shows that with the technique used any such effect is negligible, except with some obese subjects.

- 2 The elasticity of the chest-wall may allow the breast as a whole to be displaced further into the plethysmograph. This point will be dealt with in detail later.
- 3 The outflow of lymph may be retarded or reversed. There is no means of measuring this factor, but since the breast has a relatively large lymphatic drainage it is probably not negligible. However, if it may be assumed that the response of the lymph-flow to pressure-changes is not grossly unlike that of the venous blood-flow to be described, this factor cannot affect blood-flow determinations more than a very small amount.

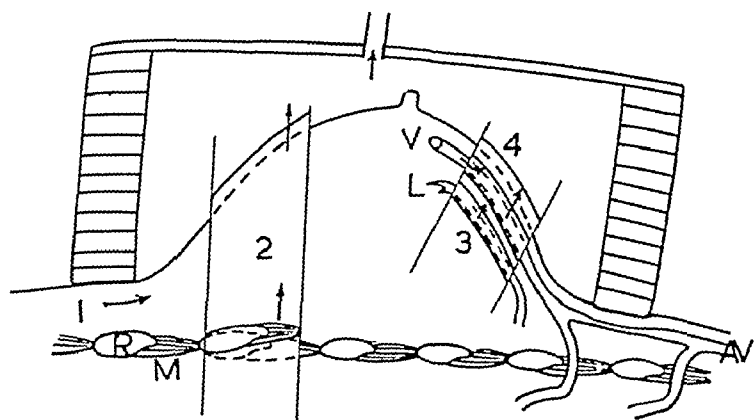


FIG 2—Possible volume changes of breast subjected to subatmospheric pressure (1-4) (see text). R rib in section. M intercostal muscle. V vein. L lymphatic. AV, anastomosing vein from adjacent region. Pectoral muscles omitted.

- 4 In so far as the suction diminishes or reverses the normal pressure-gradient driving blood along the mammary veins, blood will tend to stagnate or even to flow backwards in the veins, draining the breast, and to distend the remarkably large network of superficial veins through which almost all the blood from the gland passes. Anatomists describe valves in both the external and the internal mammary veins, but there is no doubt that when high negative pressures are applied (say 20 mm Hg) there is a considerable reflux of blood, presumably from the many anastomoses with the veins of adjacent parts.

This tendency of reduced external pressure to make blood stagnate in the superficial venous plexus is the basis of the proposed method of measuring mammary blood-flow.

It will be seen that if the appropriate subatmospheric pressure were known, it would theoretically be possible instantaneously to neutralize the normal pressure-gradient driving blood along the veins draining the

breast, so that the superficial plexus would become distended and the breast would swell at a rate equal to the arterial inflow. Since in large vessels blood behaves as an ordinary viscous liquid, smaller negative pressures than this critical one would partially inhibit the outflow in the same proportion, and the breast would swell at a corresponding fraction of the normal arterial inflow rate. It will thus be seen that the rate of

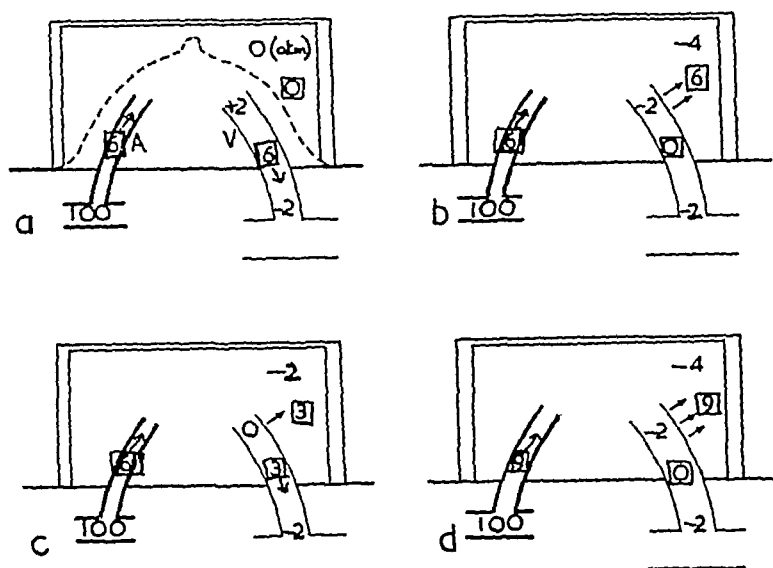


Fig 3—Diagrams to show how different plethysmograph pressures affect the rate of change of breast volume by engorgement of the superficial veins. Plain numbers thus, 100, represent pressures, and numbers in squares thus,  $\boxed{6}$ , represent rates of blood flow or change of breast-volume; all values are arbitrary. From a, b and c it will be seen that the rate of swelling of the breast is (momentarily) proportional to the amount the plethysmograph pressure is below atmospheric. Comparison of d with b shows that changes in arterial flow are reflected in the breast-volume changes under equal plethysmographic pressures, provided that the pressure gradient along the efferent veins is unchanged. The representation of artery (A) and vein (V) is not intended to be anatomically accurate.

swelling of the breast from this cause under any particular subatmospheric pressure within the range under consideration is proportional to the arterial flow, provided that variations in arterial flow do not alter the pressure-gradient in the veins, & e provided that the difference between local and central venous pressures remains constant, or almost so (fig 3).

Approximate constancy of local venous pressure in vasomotor changes results from the combined effects of the elasticity of the veins and the venomotor mechanisms. The earlier literature on this subject is summarized in Eyster's review [1926].

In short, an instrument which measures the rate of distension of the superficial veins under negative pressure is likely to reflect changes in the blood-flow through the organ

The volume-changes resulting from these four factors combined have been studied in a preliminary series of experiments. The breast of a non-lactating subject was enclosed in a plethysmograph connected to a membrane manometer, and the pressure-changes recorded following the rapid withdrawal (in  $\frac{1}{2}$  to 1 sec) of a variable volume of air. In fig 4

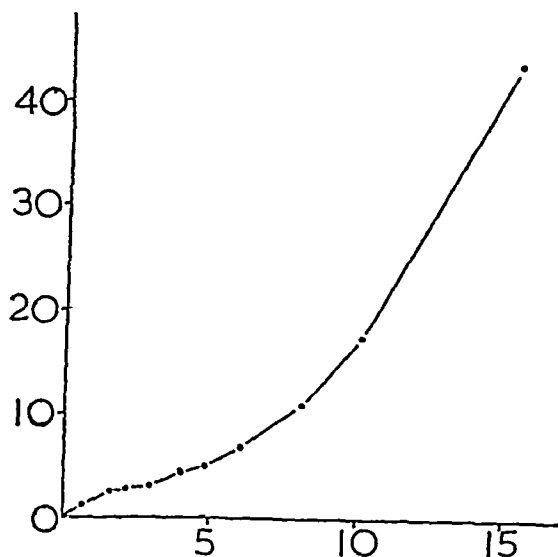


FIG 4.—Rate of swelling of breast under subatmospheric pressure (see text)  
 Abscissa—plethysmograph pressure in mm Hg below atmospheric Ordinate—  
 rate of increase of plethysmograph pressure during a 5 sec period, in mm Hg  
 per minute

are shown the results of the whole series of experiments on one subject. From the pressure-changes and the volume of the plethysmograph the volume changes can of course be evaluated. Evidence obtained later with an optical manometer, on different subjects and over a smaller pressure-range, suggests that the curve up to 3 mm Hg on the abscissa is not significantly different from a straight line, and that this line can also be extended backwards—that is, for supra atmospheric pressures—at least as far as 2 mm Hg above atmospheric. The deviations from the linear relationship at the greater subatmospheric pressures are of just the kind one would expect to result from distension of the main efferent veins by the suction from outside. If physiological conditions are to be maintained, such excessive pressures must be avoided.



## THE INSTRUMENT

The instrument consists of a breast plethysmograph and a machine by which the mass of air in it can be varied rhythmically and the consequent pressure-changes recorded. The plethysmograph (s, fig 5) is made of twelve to sixteen rings of sponge-rubber, about 8 inches diameter, 1 inch wide and  $\frac{1}{8}$  inch thick alternating with similar rings of thin card, all being cemented together and sealed with a curved Perspex

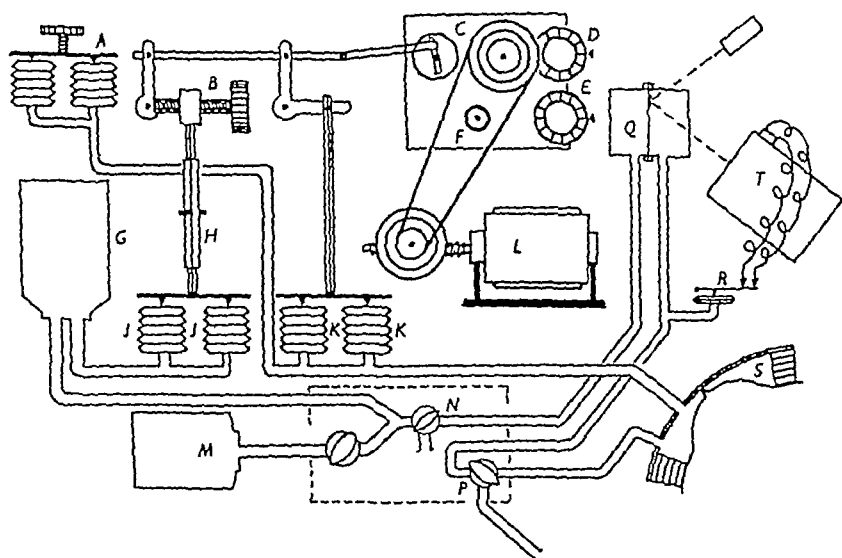


FIG 5—A, air pressure adjustment to plethysmograph, B, stroke adjustment for bellows JJ, C, variable speed and stroke crank mechanism, D, dial registering phase of cycle of events, E, dial registering stroke of crank mechanism, F, control for varying stroke of crank mechanism, G, rigid bottle, H, pressure adjustment for bellows JJ, JJ, 'compensator' bellows, KK, 'plethysmograph' bellows, L, 1/8 h p synchronous motor, M, rigid bottle used in calibration, etc., N, P, three way taps for opening tubes to air, Q, differential optical manometer, R, signal recording plethysmographic air pressure, S, special breast plethysmograph containing air, T, recording camera.

[This figure appears by kind permission of the *Journal of Physiology*]

lid. The structure is so shaped that it fits accurately on to the average female chest, completely enclosing the right breast, but nowhere touching it or apparently occluding the veins draining it. The alternation of card and sponge-rubber enables the plethysmograph to be adapted with moderate pressure to individual anatomical variations without losing more than a small part of its resistance to internal pressure-changes.

From it lead two tubes (see fig 5). One goes to a set of metal bellows actuated by a variable-throw crank mechanism, so that a volume of up to 40 c c of air is rhythmically pushed into and drawn

out of the plethysmograph, nowhere mixing with the external atmosphere. The period of this cycle can be set at either 2 sec, 5 sec, or 12.5 sec approximately.

The resulting changes in intra-plethysmographic air-pressure, modified by the effects arising from the mobile tissues of the mammary gland, are transmitted along the second tube to the differential optical manometer. Since it is convenient to study not the actual pressure-changes in the plethysmograph but the modification of such changes by the mobile tissues notably blood, the plethysmograph pressure is balanced in the differential capsule against the pressure in a dummy or "compensator" circuit. In this a set of bellows (JJ), acting synchronously with the former or "plethysmograph" set, pushes air into and out of a rigid bottle connected to the other side of the differential capsule. The stroke of these compensator bellows can be varied independently of that of the plethysmograph bellows. The pressure difference is recorded photographically on a paper moving at about 2 mm per sec, and has the form of sine-wave on which smaller waves of respiratory and cardiac origin are imposed. It is the amplitude of this main recorded wave which bears a close relationship to the blood-flow and other physiological quantities.

The instrument includes a means of compensating at will for any changes in the volume of the breast other than those induced by the pressure changes (A). It is also equipped with a signal (R), which records the periods during which the plethysmographic pressure is above atmospheric or any other selected value.

### PHYSICAL THEORY

Let  $t$  represent time, and  $f$  the frequency of the cycle of operation of the instrument, and let  $p$  represent the corresponding variable pressure in the "compensator" bellows, in  $\text{min}$ ,  $\text{min}^{-1}$ , and  $\text{mm Hg}$  respectively. Then

$$p = a \sin 2\pi ft, \quad (1)$$

where the value of  $a$  can be altered by altering the stroke of the bellows.

Now consider the pressure changes in the plethysmograph. It has been found from such experiments as those summarized in fig. 4 that, under a subatmospheric pressure, the breast swells at a rate which within certain limits is proportional to the amount by which that pressure is less than atmospheric. Moreover, the type of reasoning exemplified in fig. 3 shows that the effect of suction on the venous plexus, by making the blood partially or completely stagnate in the efferent veins, is to give just this type of volume-change. The result is that the plethysmograph air-pressure  $p'$  does not simply vary along with that in the compensator thus,  $p' \equiv 1/p$  (since the two sets of bellows

are actuated synchronously), but it also tends to rise or fall towards zero (atmospheric) at a rate proportional to its instantaneous value. These facts are expressed by the equation

$$\frac{dp'}{dt} = k \frac{dp}{dt} - bp'$$

The instrument is adjusted in a way to be described later so that  $k=1$ . The fundamental equation then becomes

$$\frac{dp'}{dt} = \frac{dp}{dt} - bp' \quad (2)$$

(It can be shown that under the conditions present the volume and pressure-changes are isothermic)

From the equations (1) and (2) we find that  $p-p'$ , the pressure registered on the record, varies as a sine-wave whose amplitude is given by the expression

$$\frac{ab}{f}(b^2/f^2 + 4\pi^2)^{-\frac{1}{2}}$$

(A constant of integration involved may be taken as zero) Since  $b$  is in fact small, the relationship is almost one of direct proportionality

The above equation holds true only so long as the strokes of the two sets of bellows are "balanced" so that the constant  $k=1$ , that is, so that but for the effects arising from mobile tissues in the breast the pressures on the two sides of the differential manometer would be continually equal. Suppose that the machine were in small degree imperfectly adjusted so that the compensator bellows gave a pressure-wave of amplitude  $ka$  where  $k \neq 1$ , other quantities remaining as before. It is now found that the amplitude of the recorded wave is

$$a \sqrt{\left( \frac{k^2 b^2 / f^2 + 4\pi^2 (k-1)^2}{b^2 / f^2 + 4\pi^2} \right)}$$

If  $k$  alone is varied, this expression has a minimal value when  $k=4\pi^2/(b^2/f^2 + 4\pi^2)$ , that is for very small values of  $b$ , when  $k=1$

#### PROCEDURE

The method of measuring  $b$  can now be followed. The plethysmograph having first been fixed in position on the supine subject, it is necessary to adjust the stroke of the compensator bellows so that the machine is "balanced," when  $k=1$ . To do this the machine is set going at the most rapid rate. Thus  $f$  is made large and  $b/f$  correspondingly small, generally into the region of 0.1 to 0.5. It will be seen from

the graph (fig 6) that, if the instrument is balanced ( $\lambda = 1$ ), the recorded wave is of very low amplitude. Or in practical terms, the air-volume changes take place so rapidly that there is no time for movement of mammary tissues, including blood, appreciably to affect the pressure-changes. In these circumstances—as will be seen from reference to the graph—a small change in the value of  $\lambda$  gives a relatively large change in the amplitude of the pressure-difference wave as recorded. The compensator stroke-volume is thus adjusted while the machine is

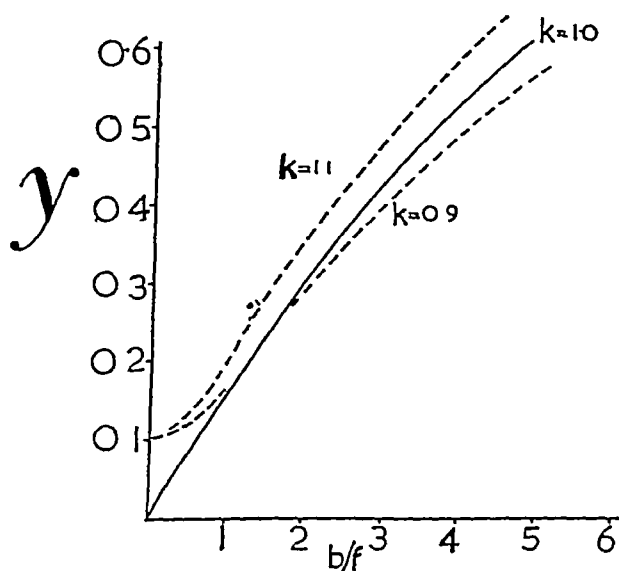


FIG 6—Graph showing amplitude of recorded wave corresponding to different values of  $b$ ,  $f$ , and  $\lambda$  (see text)

$$y = \sqrt{\frac{4\pi^2(\lambda-1)^2 + \lambda^2 b^2 / f^2}{4\pi^2 + b^2 / f^2}}$$

running, until nothing but the waves of respiratory and cardiac origin is recorded. It can then be taken that  $\lambda = 1$ .

The machine is now changed to the lowest gear. This, for the reasons described, has the effect of increasing the value of  $b/f$ , in other words, the flow of the mobile tissues now has a marked effect. Any deviations of  $\lambda$  from unity, i.e. any imbalance of the machine, now has much less part in determining the wave amplitude than has the value  $b$ . Again this will be made clear by reference to the graph.

As a check, the plethysmograph side of the manometer can suddenly be closed off, and the phase-difference between the usual wave and that due to the compensator wave alone can be determined. The relevant theoretical equations in this case are

The difference-wave follows the compensator-wave by a time  $t'$  such that

$$\tan 2\pi ft' = 2\pi bf / (k^2 b^2 + 4\pi^2 f^2 [k - 1]),$$

which when  $k - 1$  reduces to

$$\tan 2\pi ft' = 2\pi f / b$$

A record is also taken of the compensator-wave alone, to give the quantity  $a$  in terms of millimetres on the record

### DISCUSSION

The above theoretical analysis shows that the instrument will accurately record variations in mammary blood-flow provided certain conditions are fulfilled. These are

(1) That the venous pressure-gradient must be relatively unaffected by changes in blood-flow. This has already been discussed (p 221). It is quite essential that the plethysmograph must not interfere with the venous drainage, which seems to be the case in the breast.

(2) A second condition is that such factors as tissue-pressure shall not be disturbingly great. This is of course the common condition of all plethysmographic work, and palpation suggests that tissue-pressure in the breast of a non-lactating woman, or one in whom lactation is fully established, is less than that in, say, the forearm, this may not hold good in certain phases of lactation.

The displacement of the breast as a whole by the pressure-changes remains to be considered. It can be shown theoretically that any such displacement that occurs *simultaneously* with the pressure-change causing it, if it is not very great, merely has the effect of increasing the effective plethysmograph air-volume, this has been checked by the sort of experiment suggested by fig 7. On "balancing" the instrument in the way described above, it is found that the adjustment B (fig 5) has to be set as for a plethysmograph air-volume approximately twice that which in fact exists. This result is taken to indicate that this type of displacement is in fact not negligible, but it is completely accounted for by using the effective rather than the real air-volume in converting the readings into flow-rates, as described below.

Any displacement of the breast which *lags behind* the causative pressure-change cannot be separated from changes due to blood-flow. Such a factor would not be expected to vary from minute to minute in any one subject, or possibly not greatly from one subject to another. The apparent mammary blood-flow in the male subjects whose blood-flow readings are shown in comparison with other subjects in fig 8, is presumably a combination of true blood-flow in the pectoral muscles and any such lag-displacement effect. The female subjects with the

small breasts were less muscular than the male subjects, and their total blood-flow (real and apparent) was about the same. These readings

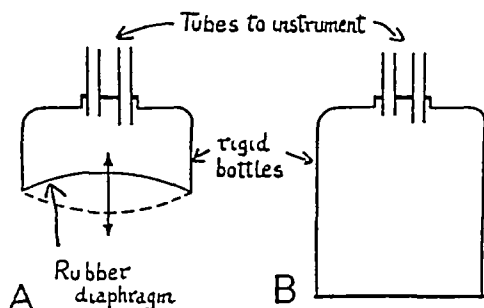


FIG 7—The arrangement A, in which the rubber diaphragm represents the elasticity of the chest wall, gives almost exactly the same effects on the instrument as the larger but completely rigid bottle B

give an approximate base-line from which true mammary blood-flow can be evaluated

The instrument gives blood-flow in terms of the quantity  $b$ . This can be converted into ml per min by multiplying it by the product of

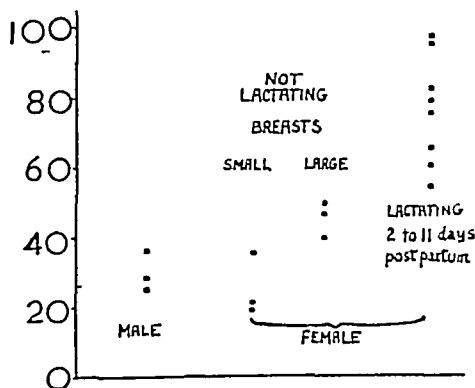


FIG 8—Rates of blood flow through the whole organ (and underlying muscle) of the first 20 subjects examined. The values are in ml of blood per min per mm Hg pressure difference along the efferent veins

the effective plethysmograph air-volume (in ml) and the pressure-difference along the veins draining the breast (in mm Hg), and dividing by the atmospheric pressure (in mm Hg). The second of these factors is not known, but is possibly in the region of unity. However, relative figures are all that are needed for most purposes.

So far no direct experimental evidence has been given that the instrument does in fact record variations in mammary blood-flow. The

physical analysis given places it beyond doubt that it is a convenient method of measuring a quantity  $b$  which in turn depends (at least partially) on the calibre of the efferent veins, and this calibre varies with the blood-flow. But can any more direct evidence be offered?

The obvious method of checking the instrument against a standard method in some organ other than the breast, or on a perfused limb,

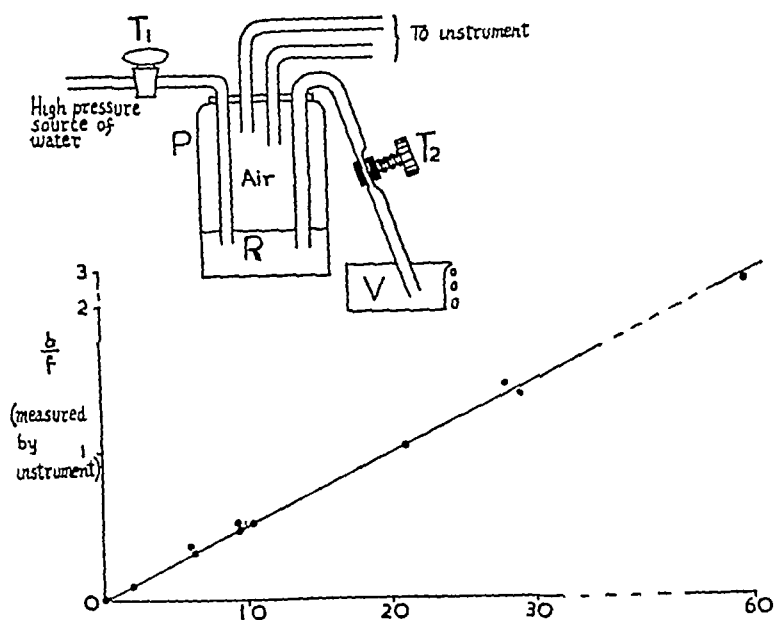


FIG 9—Experiments on an artificial circulation  $T_1$ , tap representing arterioles, P, upper part of bottle representing plethysmograph, R, water in bottle representing superficial venous reservoir of breast,  $T_2$ , tap representing effects of elasticity of veins and venomotor mechanism, adjusted until outflow equals inflow at constant pressure, V, vessel representing central venous reservoir

would demand the construction of a plethysmograph sealed on in such a way as to withstand varying pressures without interfering in the slightest degree with the venous drainage or venomotor control. This is almost a technical impossibility. If there is any such interference with venous drainage, it is found that the amplitude of the recorded wave is independent of the frequency  $f$  of the cycle of events in the instrument, and in consequence  $b$  is largely indeterminate. A direct check can be made on a simple model of the breast circulation, and the close agreement can be seen from fig 9.

Nevertheless there is much circumstantial evidence that the instrument is reliable *in vivo*. Firstly, the values of  $b$  for different subjects differ in the way that would be expected. Secondly, when the values

of  $b$  are converted into flow-rates in ml per min, the results again are not unexpected, actual figures are shown in fig 8 Thirdly, so far as they have been studied, the responses of any one subject to such influences as local cooling or inhalation of amyl nitrite are of the type that would be expected It is not the purpose of this paper to give results in detail, but fig 10 is shown as a typical example Fourthly, it is consistently found that the sine-curve drawn through the peaks of the respiratory waves has a greater amplitude than that drawn through the

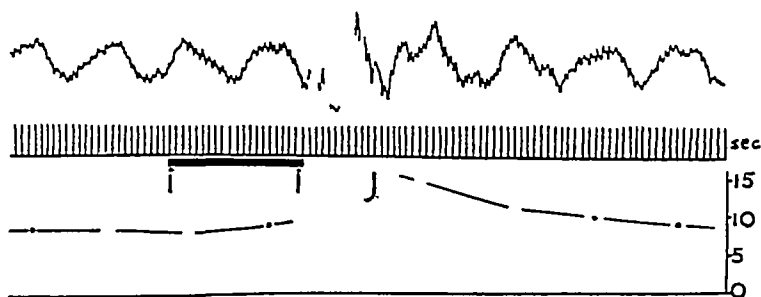


FIG 10—Record showing effect of inhalation of amyl nitrite 11, capsule held under subject's nostrils subject breathing normally 11, subject coughing Graph below shows actual height of curve in mm The heart rate and depth of respiration are seen to rise and fall *pari passu*

troughs this might mean either that inspiration is deeper during the phases of subatmospheric pressure, which seems unlikely, especially as the subject is generally unconscious of the action of the instrument, or that the blood-flow measured is greater during inspirations than during expirations Since it is the venous outflow that is in fact measured, this result accords with the known physiological facts

### SUMMARY

An instrument is described for the investigation of vascular factors in human mammary physiology, for which hitherto no suitable technique has existed It involves a modification of the plethysmographic method

### ACKNOWLEDGMENTS

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# INITIAL RESPIRATORY RESPONSES TO THE INTRA-TRACHEAL INHALATION OF PHOSGENE OR AMMONIA

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RESPIRATORY responses to the intratracheal inhalation of lung irritants have been described by many workers since the time of Paul Bert Laqueur and Magnus [1921], and more recently Newton [1941], Fegler [1942], Smythe [1943], and Whitteridge [1948], have described some of the more immediate effects of phosgene inhalation seen in the cat. In the experiments described in the first part of this paper we have studied the action of phosgene on anaesthetised cats and dogs and compared it with that of ammonia, which is a typical respiratory irritant capable of initiating prompt and characteristic changes in the breathing. After analysis of these experiments it seemed desirable to determine whether there was any direct action on the lungs by these gases which, apart from the stimulation of afferent systems of the animal, might influence the total response. Experiments specially designed for this purpose were carried out on isolated blood-perfused dog lungs and are described in the second part of this paper.

## METHODS

### *Anaesthetics and Operative Procedures*

Mixtures of chloralose 0.05 g and urethane 0.5 g/kg given subcutaneously were used for anaesthesia in the phosgene experiments. In the ammonia series dogs were given either the chloralose and urethane mixture, or nembutal (0.032 g/kg) or sodium barbital, cats were given nembutal 0.032–0.06 g/kg, and rabbits 0.032 g/kg. The barbiturate anaesthetics were given either intraperitoneally (dogs and cats) or intravenously (rabbits). So far as we could judge the type of anaesthetic was not a factor which decisively modified the results.

All animals were tracheotomised. One group of five cats had their lungs chronically sympathectomised (bilateral removal of the stellate and T 1–4 ganglia) in a two-stage operation four and three weeks before

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experiment In acute experiments stelletomy was performed either through the neck or between the 3rd and 4th intrathoracic spaces Another series of tests was carried out on animals with chests opened by a mid-line split down the sternum, in such cases the stellate ganglia were removed through the sternal opening

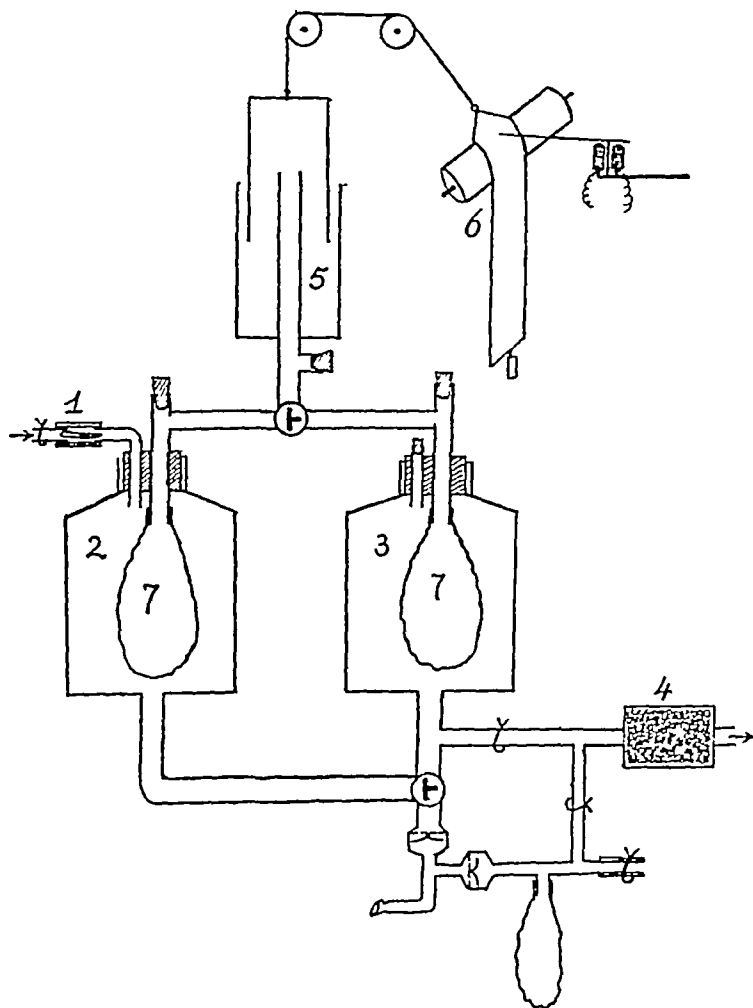


FIG 1—Gassing and respiratory recording apparatus (1) Rubber tubing containing an ampoule of liquid phosphene After the ampoule is broken the gas is washed into the container (2) with compressed air Either the gas container (2) or the pure air container (3) can be connected to the respiratory system at will by manipulating three way taps Capacity of containers 20 litres capacity of rubber bags (7), 6 litres (4) absorber with charcoal (5) smoked paper strip connected to the bell of the spirometer (5), 30 second time intervals were marked on this paper strip by an electromagnetic signal

*Gassing—Phosgene*—Phosgene was administered intratracheally for periods of up to 30 minutes via an inspiratory valve from a closed system of bottles with compensatory rubber bags arranged so that the gas mixture inhaled was of nearly constant composition (see fig 1) Sealed ampoules of liquid phosgene provided by the Ministry of Supply were used Gas concentrations (vol/vol) were calculated to have ranged from 1/500 to 1/250 in tests on dogs (20 animals) and from 1/1000 to 1/500 in tests on cats (24 animals) In calculating the concentration no allowances are made for the amounts of phosgene which may have been destroyed in contact with moist rubber and metal parts of the apparatus

*Ammonia*—Ammonia was given by various methods One was to saturate a cotton-wool swab with ammonia solution (B P S G 0 888) and hold it against the tracheal cannula for a given time (1–18 sec) during spontaneous respiration (Tests were made on 12 dogs, 3 cats and 10 rabbits) Another was to admit an air-ammonia mixture to a closed respiratory system incorporating either inspiratory and expiratory valves, or a carbon dioxide absorber (soda-lime) connected directly to the tracheal cannula The mixture was prepared by equilibration of air with a strong solution of ammonia at room temperature and pressure The test mixture was contained in a bottle, and it was either drawn into the ventilating system simply by opening communicating taps, or was displaced by a syringe into the trachea through a thin catheter in which case the latent period of response was reduced The volumes administered varied from 20 to 30 ml for dogs, 4 to 20 ml for cats and 20–90 ml for rabbits Air-ammonia mixtures were administered in both of these ways to animals breathing spontaneously, or to animals with open chests and being ventilated by a Starling Ideal pump

## RECORDS

*Arterial blood pressure* was measured with a mercury manometer, and sometimes also with a membrane manometer, from the femoral artery of cats and dogs, and from the carotid artery in rabbits

*Chest movement, rate and volume of respiration* were recorded as follows (a) *respiratory minute volume* by a spirometer (see fig 1, phosgene experiments), (b) *tidal air volume* by a small bell spirometer or by a large capacity tambour, (c) *chest movements* by a corrugated rubber tube stethograph (recorded in nearly all experiments), (d) *intratracheal pressure changes* by a sensitive Marey tambour (recorded in the ammonia experiments with open chests), (e) *intrathoracic oesophageal pressure changes* by a Marey tambour

The last of these measurements (e) provided a close approximation to the *intrapleural pressure changes*, this was checked by taking records simultaneously of both pressures in some experiments Spontaneous

waves resulting from the contraction of the œsophagus rarely occurred, and when they did, were quite irregular and easily distinguished from the rhythmical respiratory fluctuations

Changes in intrapulmonary resistance occurring during the course of the experiment were obtained by calculating the ratio *excursion in mm of thoracic œsophageal pressure/excursion in mm of stethograph* Tidal air-volumes, when available, were used instead of the stethograph readings (Christie [1938] used a method analogous to this for the assessment of changes in the resistance of human lungs) Control tests with inhaled CO<sub>2</sub> and intravenous injection of acetylcholine showed that the former caused no change or a reduction in the ratio, while the latter agent produced a sudden increase in ratio These effects indicated that changes in the ratio did give a reliable measure of changes in bronchial resistance

## RESULTS

*Dogs*—The inhalation of either phosgene or ammonia caused a typical somatic motor response after a latent period of 1–6 seconds in the majority of animals This consisted of a pronounced respiratory pause of 6 to 48 seconds followed by a period of very rapid shallow breathing (depth decreased 20 to 50 per cent, rate increased 100 to 200 per cent (see fig 2)) In response to ammonia, the changes in rate and depth were somewhat less consistent than those due to phosgene, a fact possibly attributable to greater variations in gassing technique The results have been briefly summarised in Table I

In addition to the somatic motor response, increases in the intrapulmonary resistance indicated that bronchoconstriction always occurred within 1½–5 minutes from the beginning of exposure An earlier increase, complete within 1 minute, was also but not so consistently seen When produced by ammonia inhalation, the late increase in intrapulmonary resistance had always reached its peak and begun to subside within 10 minutes, but with phosgene, similar recovery occurred in less than 50 per cent of the animals tested

After section of both cervical vagosympathetic trunks, somatic motor responses could still be elicited by either phosgene or ammonia, but with the loss of certain components this assumed quite a different character The apnoea (apnoea is used here in its strictest sense as cessation of breathing) and shallow respiration, characteristic of the response of dogs with intact vagi, were no longer seen, instead the reaction consisted of a marked increase in respiratory rate, a slight increase in respiratory depth, and a lengthening of the latent period which might now be of the order of two minutes In a more detailed investigation of this reaction, using ammonia as the test gas, it was found that no further modification was produced by denervation of the carotid sinuses, but it was suppressed by removal of the stellate ganglia

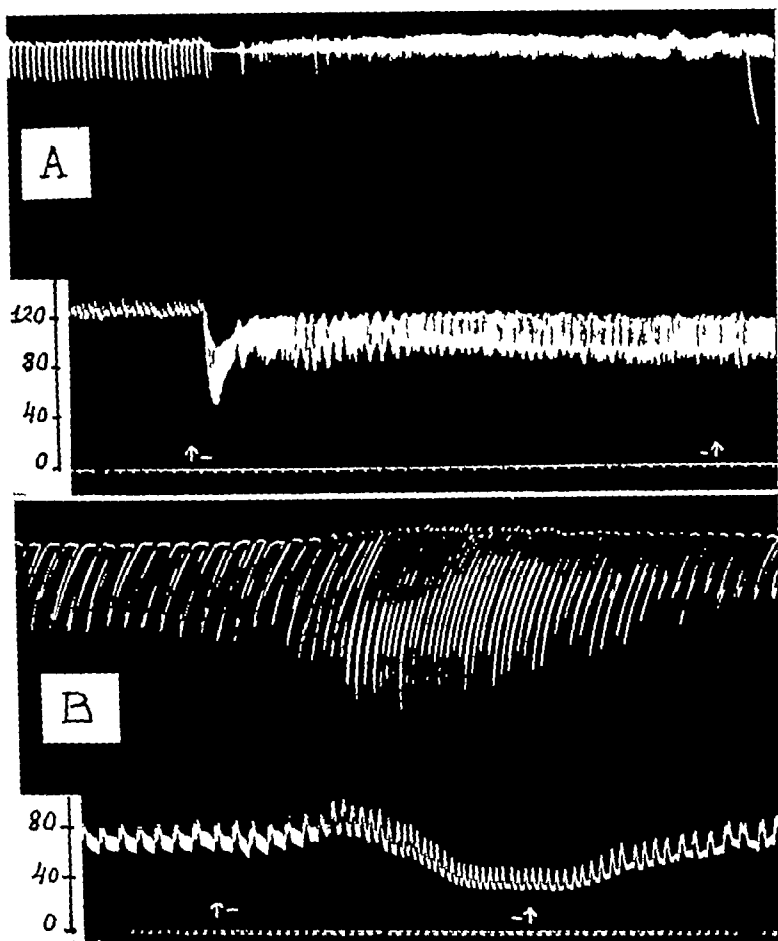


Fig 2—A The initial respiratory reaction of a 5.3 kg dog (with normal lung innervation and chlorolose urethane anaesthesia) to phosgene inhaled in concentration of 5.0 g/cubic metre  
*Upper tracing* Intra oesophageal pressure variations  
*Lower tracing* Carotid blood pressure in mm Hg  
*Time* 6-second intervals

B The respiratory reaction of 9 kg dog (chlorolose urethane anaesthesia and both vagosympathetic trunks sectioned) to phosgene exposure (in a concentration of 5.0 g/cubic metre)  
 Records as in A

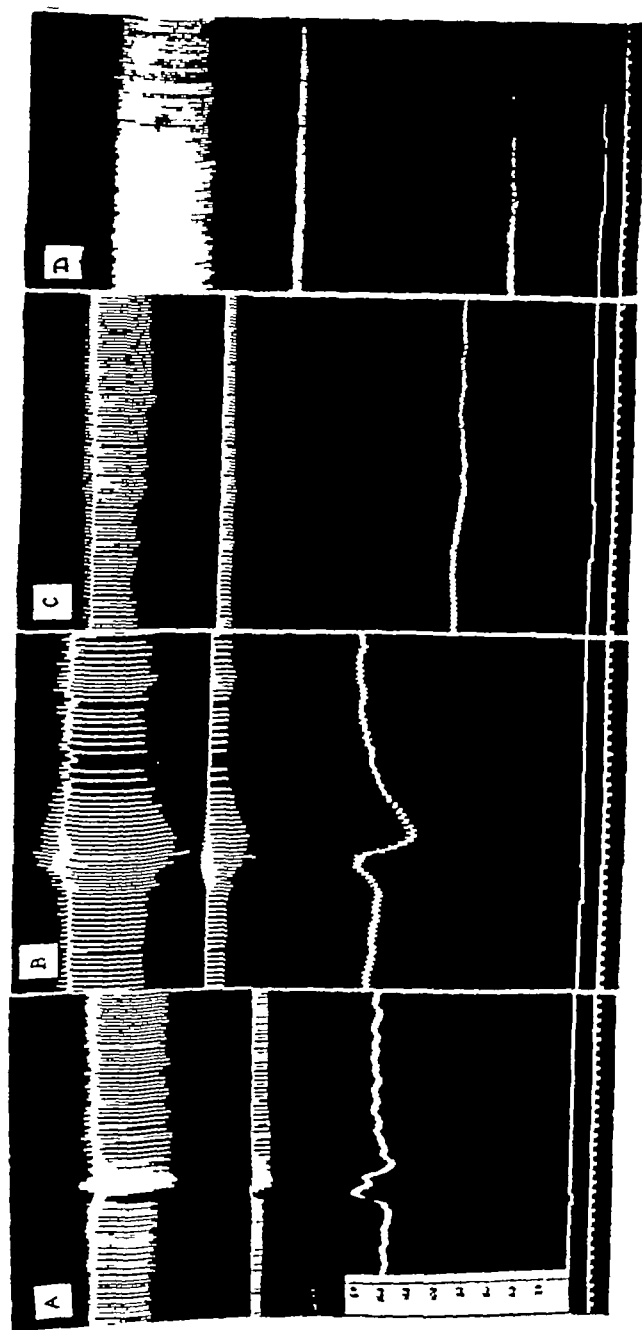


FIG. 3.—The initial respiratory response of a dog under nembutal to inhalation of ammonia vapour (Method 1)  
 A Normal innervation  
 B After both stellate ganglia removed  
 C After both vagosympathetic trunks sectioned  
 D Same as C, repeated 35 minutes later  
 Upper tracing Intra oesophageal pressure variations  
 Middle tracing Stethograph record of chest movements  
 Lower tracing Femoral arterial blood pressure in mm Hg



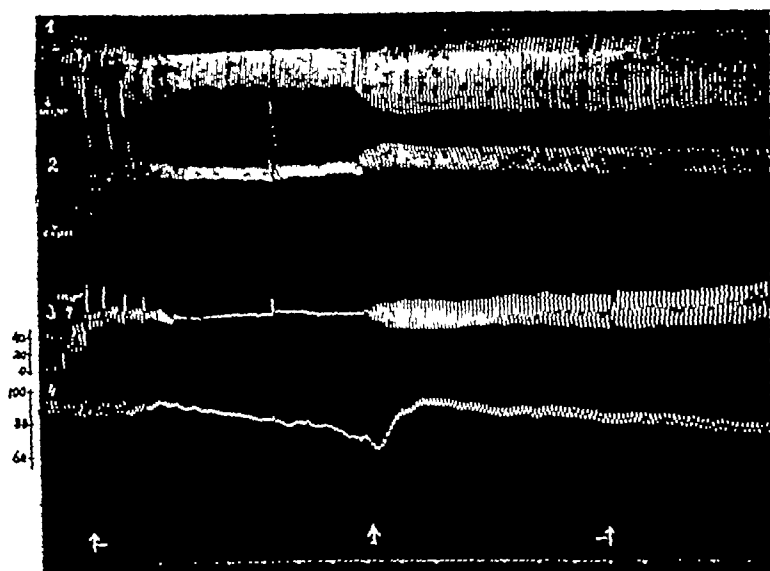


Fig 4—The initial respiratory reactions of a cat (2.8 kg chlorolose urethane anaesthesia) exposed to phosgene in a concentration of 5 g/cubic metre, showing the effect of section of both vagosympathetic trunks during poisoning

Tracings 1 Intra oesophageal pressure variations  
 2 Stethograph record of chest movement  
 3 Tidal air volume in cubic centimetres  
 4 Femoral arterial blood pressure in mm Hg

Time 30 second intervals

At first arrow phosgene exposure begun At second arrow section of both vagosympathetic trunks At third arrow exposure stopped

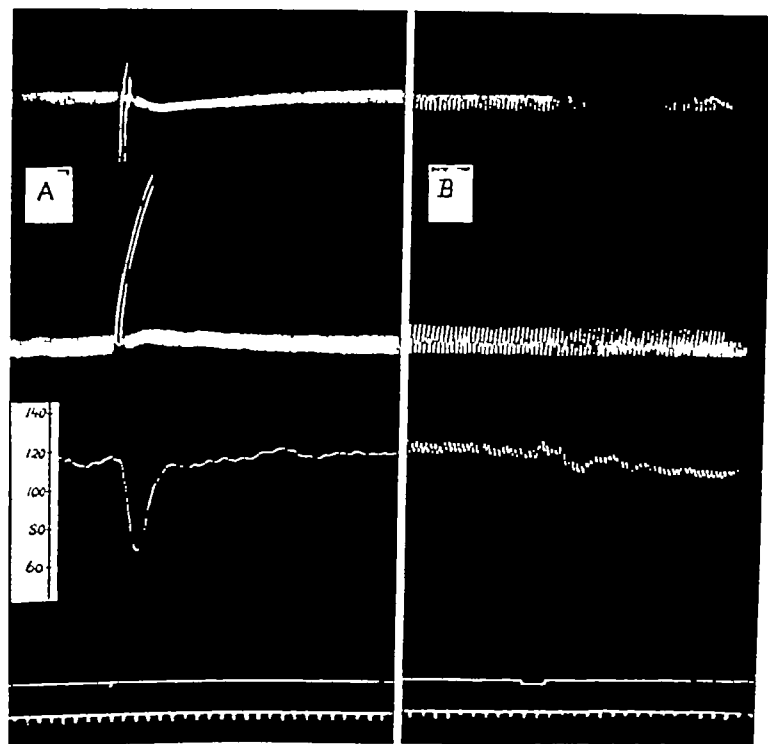


FIG. 5—The respiratory responses of a 2.8 kg rabbit (nembutal) to inhalation of ammonia vapour (Method 1)

A Normal lung innervation

B After section of both vagal trunks

*Upper tracing* Intra-oesophageal pressure variations

*Middle tracing* Stethograph record of chest movements

*Lower tracing* Carotid arterial blood pressure in mm Hg

*Time* 6 second intervals

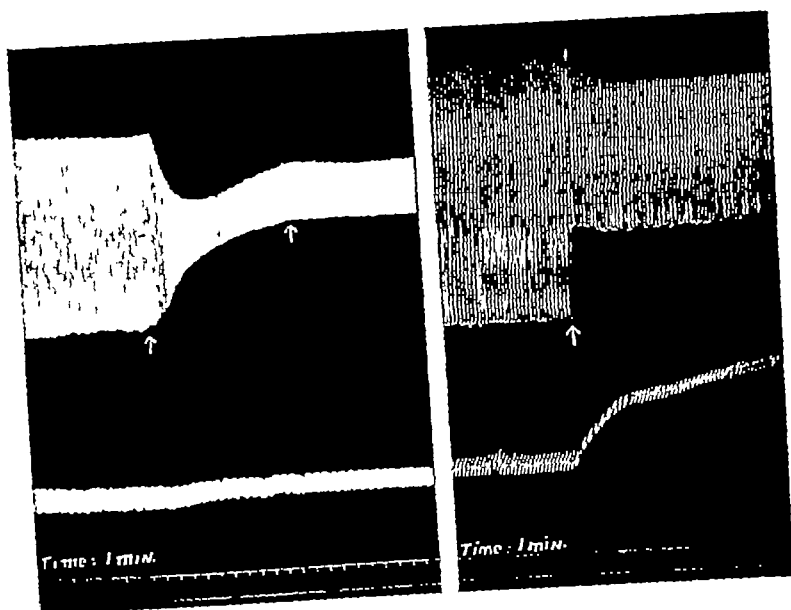


FIG 6—A Experiment 72 Dog (Separated isolated perfused lungs)  
 Upper tracing Right lung tidal air (negative pressure ventilation  
 0.4–10 cm H<sub>2</sub>O)  
 Lower tracing Right pulmonary arterial pressure 12.4 mg  
 (24 c.c. air gas mixture) phosgene was given between signals, in  
 successive doses of 0.25 mg per inspiration. Concentration,  
 1.89, initially rising to 7.6 g/m<sup>3</sup> finally  
 Maximum response after 5 minutes of gassing  
 B Experiment 52 Dog (Separated isolated perfused lungs)  
 Upper tracing Right lung tidal air (negative pressure ventilation  
 -0.4 to -10 cm H<sub>2</sub>O)  
 Lower tracing Right pulmonary arterial pressure  
 At signal, 100 mg phosgene given as a single dose at the beginning of  
 inspiration.  
 Approximate concentration = 43.5 g/m<sup>3</sup>

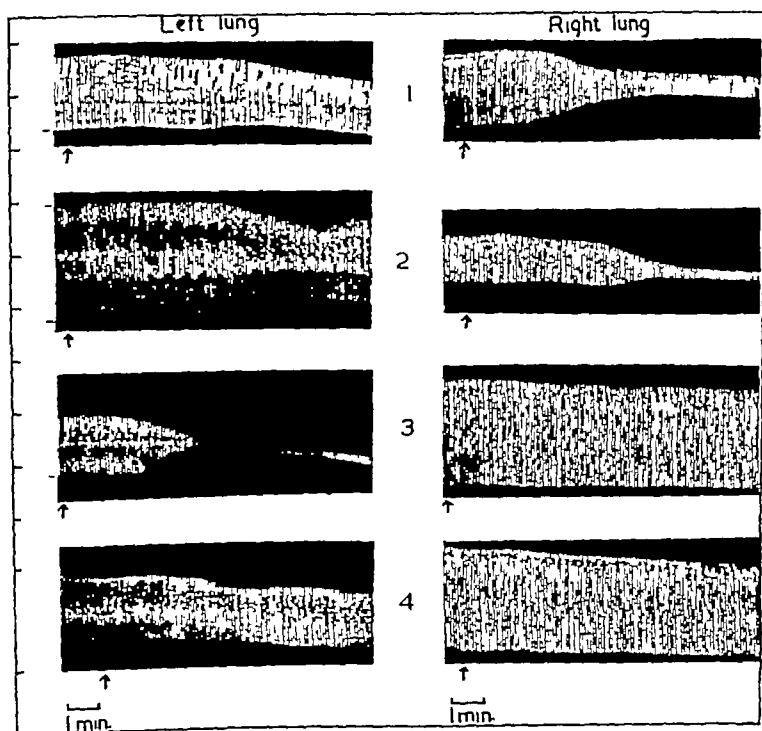


FIG 8—This illustrates the potentiating action of eserine on the bronchoconstrictor response to ammonia and suppression of this potentiation by atropine

In this experiment the two lungs were separately ventilated and perfused

- 1 At 12 01 p.m. eserine was added to the right lung perfusate so that its final concentration was 1/200,000. At 12 08 p.m. (arrows) 7.5 c.c. of air ammonia vapour were given to each lung
- 2 At 1 10½ p.m. (arrows) 7.5 c.c. of air ammonia vapour were given to each lung but 10 mg. of nicotine had been previously added to the right lung perfusate
- 3 At 2 34 p.m. eserine was added to the left lung perfusate so that the final concentration was 1/200,000. 2 mg. atropine had been previously added to right lung perfusate. At 2 41 p.m. (arrows) 7.5 c.c. air ammonia vapour were given to each lung
- 4 At 3 43 p.m. (arrows) 7.5 c.c. air ammonia vapour were given to each lung. Both perfusates now contain eserine, atropine and nicotine

(Each ordinate division represents 40 c.c. tidal air)

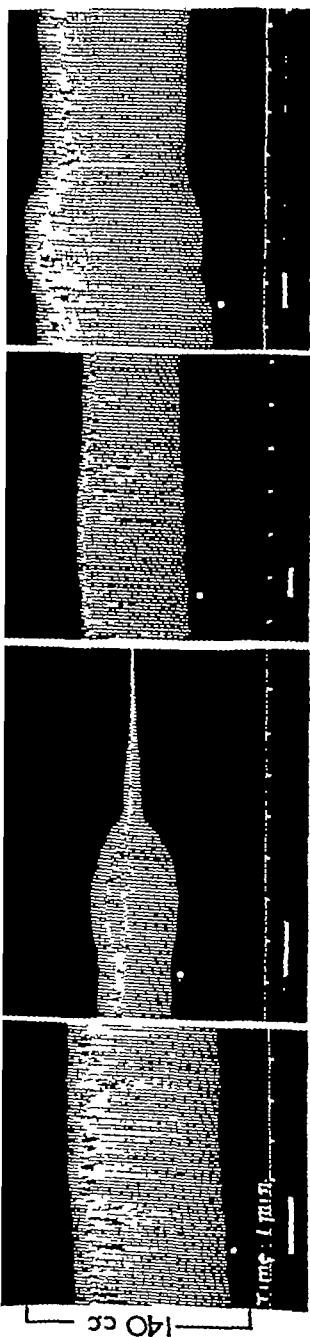


Fig 9 —Illustrates the inability of atropine to protect against a large dose of air ammonia vapour (The lungs were perfused and ventilated as a single unit) Tidal air responses only are shown

- 1 At signal (11 54 p m ) 8 c c air ammonia vapour inhalation
- 2 At 12 29 p m. eserine given via the bronchial artery (2 mg ) and the pulmonary perfusate (2 mg )
- 3 At signal (12 48½ p m ) 8 c c air ammonia vapour inhalation (Note the slight dilatation before onset of constriction )
- 4 At signal (2 13½ p m ) 8 c c air ammonia vapour inhalation after previous addition of atropine to the bronchial artery (2 mg ) and to the pulmonary perfusate (2 mg )
- 5 At signal (3 15 p m ) 20 c c of air ammonia vapour inhalation Thus time there is quite a marked constrictor response

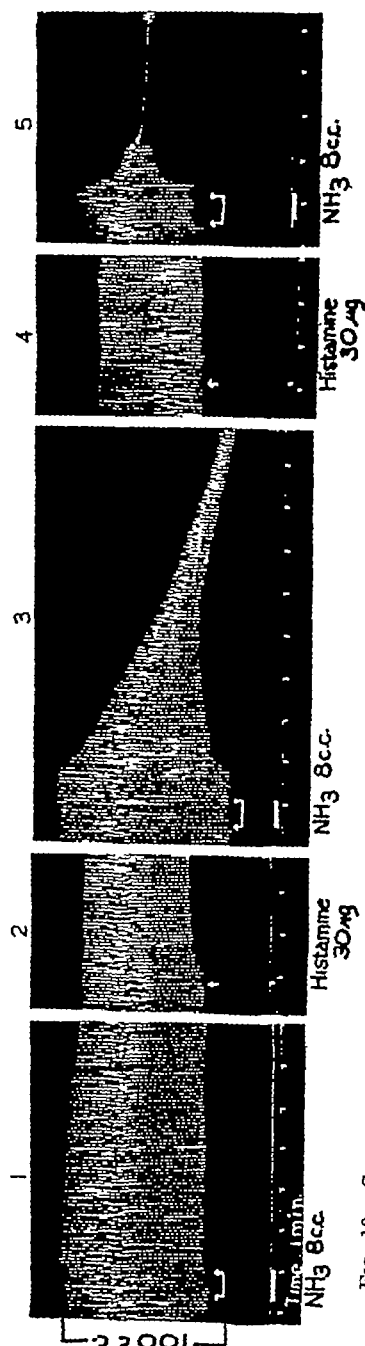


Fig 10 —Comparison of the tidal air response to injected histamine and inhaled air ammonia vapour (isolated perfused dog lungs) Between 2 and 3 p m eserine was added to the perfusate to give a final concentration of 1/200,000 There is no potentiation of the histamine response



TABLE I—INITIAL RESPIRATORY RESPONSES OF TRACHEOTOMISED DOGS TO INHALATION OF PHOSGENE OR AMMONIA

Innervation of lung	Irritant	Latent period in seconds	Apnoea	Respiratory rate		Respiratory depth		Total number of animals	Intrapulmonary resistance		
				Increase	Decrease	Increase	Decrease		1st rise	2nd rise	Total number of observations
Intact	{ Phosgene Ammonia }	3-5	10	12	0	1	12	13	3	11	13
		1-6	13	13	2	3	9	16	4	4	4
Vagotomised	{ Phosgene Ammonia }	20-120	0	4	0	5	0	7	0	5	7
		1-20	0	16	0	15	0	17	0	5	5
Vagotomised and sympa- theotomised	{ Ammonia only }	42-72	0	2*	0	2*	0	6	0	2	2

\* The effects were very slight, and coincided with a fall in blood pressure

(The figures given for each type of response represent the number of individual animals in which each type of response was observed. Intrapulmonary responses were not measured in all cases, and the total number of observations in this case is therefore given in the final column.)

(see fig 3) These results indicated that in the dog some afferent pathways concerned in the somatic motor response to respiratory irritation reach the central nervous system via the stellate ganglia, and in thus implicating an extra-vagal afferent pathway for such responses, were in agreement with the results of Craigie [1922], Cromer, Young and Ivy [1933], and Fegler [1933]

In contrast to the reflex somatic motor responses, the delayed increase in intrapulmonary resistance seen after gassing persisted in spite of division of the vagosympathetic trunks and removal of the stellate ganglia. Some attempts to prevent it by atropinisation of the animal were inconclusive. On the other hand, the early increase in intrapulmonary resistance sometimes seen when the vagi were intact was never present after vagal section.

Inhalation of phosgene also caused a sharp fall in blood pressure which occurred within three minutes from the start of exposure. After section of the vagosympathetic trunk the effects of phosgene on the blood pressure were less consistent: a fall occurred in 2 dogs, a rise in 2 others, and no change occurred in 3. Ammonia produced a fall in blood pressure of 20 per cent or less in 3 and a rise in 5 out of a total of 12 dogs. After division of the vagosympathetic trunk, ammonia caused some change (either a rise or a fall) in only half the animals tested, but when the stellate ganglia were removed as well it consistently caused a fall.

*Cats*—Cats, when exposed to phosgene or ammonia, showed a marked acceleration and decrease in depth of respiration. The apnoea, so regularly observed in dogs, occurred in only 1 out of 28 cats. A very characteristic feature of the response was a shift of the expiratory level of the chest towards the inspiratory position (see fig 4), an effect observed in only 3 out of 29 dogs. Whitteridge [1948] reported analogous findings in cats exposed to lower concentrations of phosgene, with evidence of an increased electrical activity of the diaphragm throughout expiration. The increase in the intrapulmonary resistance on inhalation of either gas, previously mentioned in connection with dogs, was also clearly marked in cats. An early increase occurred within one minute from the start of exposure, while a later increase occurred within five minutes. The results have been summarised in Table II.

Section of the vagosympathetic trunks totally suppressed the initial somatic responses to ammonia and phosgene in the cat, and there was no evidence of a residual reaction as observed in dogs after vagotomy. Slight transient changes in rate and depth of respiration were sometimes recorded, but such changes probably originated from stimulation of the carotid sinus area (unpublished observations). The fact that the shift in the expiratory level of the chest disappeared after vagal section was of interest in view of Smythe's finding [Smythe, 1943] that bilateral



TABLE II — INITIAL RESPIRATORY RESPONSES OF TRACHEOTOMISED CATS TO INHALATION OF PHOSGENE OR AMMONIA

TABLE II —INITIAL RESPIRATORY RESPONSES OF TRACHEOTOMIZED ANIMALS									
Innervation of lung	Irritant	Latent period in seconds	Inspiratory shift of thorax	Increased respiratory rate	Respiratory depth		Total number of animals	Intrapulmonary resistance	
					Decrease	Increase		1st rise	2nd rise
Intact	{ Phosgene Ammonia	3-30	14	14	15	0	15	5	5
		1-20	12	10	6	3	13	10	9
Vagotomised	{ Phosgene Ammonia	6-120	0	2*	0	1	4	0	4
		9-50	0	6*	2*	3*	15	0	11
Vagotomised and symplectothecomised	{ Phosgene Ammonia	6-120	0	4*	0	4*	5	0	5
		9-50	0	1*	0	0	6	0	5

\* Effects were very slight indeed

\* Effects were very slight indeed  
 (The figures given for each type of response indicate the number of individual animals in which such a response was observed  
 Note that intrapulmonary responses were measured in only a proportion of the experiments, the total number of such observations being indicated in the final column )

vagotomy did not affect the increased negativity of the intrapleural pressure which followed exposure to phosgene

The early increase in intrapulmonary resistance following inhalation of either irritant no longer occurred after vagotomy, but as in dogs, the late increase was still present in 15 out of 16 animals and was, if anything, more pronounced. Further, partial or complete sympathetic denervation of the lungs did not appear to influence its development

Following phosgene inhalation the blood pressure either showed a fall (5 animals) which occurred within three minutes or no change at all (2 animals). After ammonia a fall in blood pressure occurred in 3 animals, a rise in 2 and no change in 1 animal. Responses to either gas obtained after vagotomy or vagotomy and stellectomy were equally variable

*Rabbits* —The effects of ammonia vapour on the respiratory responses of rabbits were also observed. The initial reaction differed in some respects from that already described for dogs and cats. After a latent period of only 1 second (concentration of vapour was relatively high in these experiments), all of a total of 10 animals gave a deep cough, which in two cases was followed by brief apnoea. Increased rate of respiration then followed in 6 animals and a decreased rate in 4, simultaneously there was a decrease in depth of breathing in 4 animals and an increase in depth of breathing in 3. Fig 5 illustrates a response in which increased rate and decreased depth occurred. The responses in rabbits resembled those in cats, in that a shift of the expiratory level of the chest towards the inspiratory position was seen in all animals, while apnoea was relatively infrequent. We were only able to detect an increase in intrapulmonary resistance in response to gassing in 1 of 5 animals

The cough, the shift of the thorax, and the changes in rate and depth of breathing were all eliminated by bilateral vagotomy. This finding supports the belief of Larsell that only the vagus nerve conducts sensory impulses from the lungs of rabbits

The arterial blood pressure dropped sharply by about 40–50 per cent on inhalation of ammonia. This was probably connected with the violent cough, since after vagotomy there was instead a gradual fall of blood pressure of 20 per cent or less

## DISCUSSION

On analysis of our results we find that in dogs and cats the initial irritant action of high concentrations of phosgene is similar to that of ammonia, and this suggests that the same pulmonary receptors are sensitive to the two substances. There is, however, a species difference in the cat, inasmuch as the afferent pathways chiefly concerned travel in the vagus, while in the dog extra-vagal pathways also exist which may be interrupted by removal of the stellate ganglia

The respiratory responses of the two species do not correspond in detail. Apnoea, which is prominent in the dog, does not occur in the cat, while, on the other hand, the shift of the expiratory level of the chest towards inspiration has been observed in the cat and the rabbit but not in the dog. It is possible that this latter difference is more apparent than real and depends on the method of measurement. The thorax of the cat is more elastic than that of the dog, and in this case the stethograph may reflect more accurately the volume changes in the thorax.

Those features of the respiratory response which are common to ammonia and phosgene in both species are the rapid shallow breathing and the early and late increases in intrapulmonary resistance. There are various indications that both of these increases in intrapulmonary resistance are due to bronchoconstriction. For its rapid development the vagi must be intact, but a more slowly developing response is still present after the extrinsic innervation of the lungs has been severed. The most probable explanation of this, is that both phosgene and ammonia act directly on peripheral structures to produce a motor response which is independent of those mediated by the central nervous system.

This conclusion might be open to certain objections if it were based only on the results so far presented, but it is supported by the experiments of Daly, Eggleton, Elsdon and Hebb [1946], who found that phosgene causes bronchoconstriction in the isolated perfused lungs of dogs, and by Laqueur and Magnus [1921], who demonstrated the same responses to high concentrations of phosgene in the isolated perfused lungs of cats. In the experiments now to be described we have attempted to analyse the effect of phosgene on the isolated dog lungs more carefully, and have compared the action of this gas with that of ammonia.

### *Experiments on Isolated Perfused Dog Lungs*

Experiments were performed on separated isolated blood-perfused lungs of dogs kept under negative pressure ventilation according to the method of Daly, Hebb and Petrovskaja [1941], as modified by Hebb and Nimmo Smith [1945]. By this technique the right and left lungs were set up each with an independent circulation and ventilating system. In the majority of the phosgene experiments, tests were carried out on one lung only, the other being treated as a control. In other experiments, tests of either phosgene or ammonia were carried out on the two lungs often simultaneously, when some other difference of treatment had been introduced (*e.g.* intravascular injection of drugs). In addition, seven experiments were performed in which both lungs were perfused and ventilated as a single unit. In five of these, injections were made into the main right posterior bronchial artery through a

cannula tied into the parent (intercostal) artery as described by Berry, Brailsford and Daly [1931] This method was used, because drugs injected by this route will reach parts of the bronchial tissue and intrapulmonary nerves and ganglia which may not be supplied by the pulmonary arterial system

*Administration of Gases* —The lungs enclosed in the negative pressure chamber were connected externally to recording spirometers, the whole forming a closed system For the purpose of gassing, a glass side-arm on the bronchial (or tracheal) cannula could be put in communication with a glass syringe, from which the gases were injected into the airway Phosgene was given either as a large single injection (up to 10 mg) of the pure gas, or in small successive injections in step with each respiration When low concentrations were required the latter method was used, the gas being diluted with air as required and injected in small volumes at the end of each expiration, so that it could be diluted and carried into the lungs with the volume of air entering them at the succeeding inspiration The concentration was therefore dependent upon the tidal air volume during injection and has been calculated as such The advantages of the method were that the dead space was small (ca 3 to 5 ml), and that the recording of tidal air was not interrupted by gassing

For injection, ammonia vapour was withdrawn into a syringe from the top of a full Winchester of ammonia (S G = 0.888, B P), and was thus diluted with air to an extent depending upon temperature and other factors By analysis we found that approximately half the total volume in the syringe consisted of ammonia vapour For convenience we have expressed the dosage throughout in terms of the total volume of air-ammonia mixture injected

Since the lungs were ventilated by extrapulmonary negative pressure variations of constant magnitude (ca  $-0.4$  to  $-9$  cm  $H_2O$ ), bronchomotor responses were shown by changes in the tidal air, a reduction indicating bronchoconstriction and an increase bronchodilatation These changes have been expressed as percentages of the existing control values, in order to compare quantitatively the responses of different lungs which varied widely in respect of their normal ventilating capacity After experiment the lungs were examined for evidences of oedema, congestion and tissue hæmorrhage Macroscopic observations were checked histologically in most cases The area of tissue supplied by the bronchial vessel injection route was assessed by the injection of Indian ink (in saline) at the end of perfusion

## RESULTS

(a) *Phosgene* —In confirmation of Daly *et al* [1946], we found that phosgene produced bronchoconstriction in the isolated perfused lungs,

and it was in fact observed in all of 50 experiments carried out in this laboratory (including some which will be reported elsewhere) The most remarkable feature of the response was its brief latent period With concentrations of 0.6 to 1 g/m<sup>3</sup>, a measurable reduction in tidal air occurred within a few respirations At higher concentrations it was almost synchronous with the passage of the gas into the lung, and the reduction in tidal air was evident in the reduced volume of the expiration which followed The rate at which the response developed to its maximum could largely be controlled by the rate of admission of the gas to the airway, as may be seen by comparing the two tracings of fig 6 In one, approximately 100 mg of phosgene was introduced into the airway as a single dose, and the resulting bronchoconstriction had reached its peak within a few respirations In the other, gassing was carried out with relatively small doses added during several successive respirations, and here instead of an abrupt reduction in tidal air, the response took a gradual course in step with the gassing until it reached a maximum

The sensitivity of the lungs to the bronchoconstrictor action of phosgene was subject to large individual variations In nine experiments single doses of 100 mg produced changes in the tidal air varying from -20 to -69 per cent Given in small repeated doses, the range of response representing the maximal effect of the gas on the individual lungs was from -20 to -80 per cent, when the total amounts of phosgene given varied from 8 to 20 mg We ascertained that the response was maximal in these experiments by continuing the administration of phosgene until the reduction in tidal air ceased

Inhalation of phosgene sometimes produced a rise in pulmonary arterial pressure The response was largest when single doses of phosgene were given (see fig 6), though it was not always present With small repeated doses of phosgene a slow rise in pulmonary arterial pressure frequently occurred, but was often not of a significant order (less than 5 per cent in 5 to 15 minutes), it was sometimes absent or replaced by a depressor effect It seemed probable that the changes in blood-pressure observed were largely due to changes in intrapulmonary pressure arising from the associated bronchoconstriction [Daly and Hebb, 1942], and that phosgene did not have a direct vasomotor action The absence of any marked change in pulmonary arterial pressure also made it improbable that the rapid reduction in tidal air was of vascular rather than bronchomotor origin Measurements of the volume in the venous reservoir showed that changes in the lung blood-volume, even when tidal air changes were maximal, could not have exceeded  $\pm 3$  cc, if present at all

These remarks refer to the results of single tests carried out on 50 isolated lung preparations The quantitative assessment of the response was handicapped by the fact that the administration of phosgene was

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## RESULTS

(a) *Phosgene* —In confirmation of Daly *et al* [1946], we found that phosgene produced bronchoconstriction in the isolated perfused lungs,

preceded the bronchoconstriction. In addition, unlike phosgene, ammonia in the doses tested did not cause oedema, nor did it produce histologically evident bronchial desquamation, an effect which is characteristic of phosgene-treated lungs [see Daly *et al*, 1946]. Further, in these isolated lungs ammonia did not appear to promote mucous secretion. This may be related to the fact that the bronchial vessels

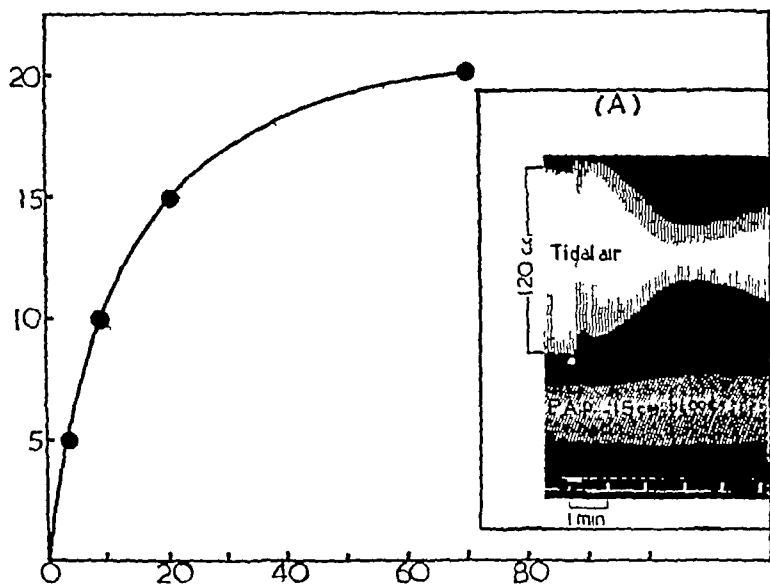


FIG. 7.—The response of an isolated perfused dog lung to graded doses of air ammonia vapour

Ordinate c.c. of inhaled vapour

Abcissa percentage reduction in tidal air

Inset A shows the actual effect produced by 20 c.c. of vapour and corresponds to the final point on the graph. Note the early onset of spontaneous recovery. The mechanical effect of tidal air reduction on the pulmonary arterial pressure is also well illustrated.

in these isolated lungs were not perfused except through back-flow of blood from the pulmonary vessels.

The administration of ammonia was followed by slight changes in pulmonary arterial pressure in some tests. There might be a rise, or a fall, in the mean pressure, accompanied by an increase in pulse pressure. None of the changes observed suggested anything other than a mechanical effect secondary to the bronchoconstrictor action of ammonia vapour. We should also note that it did not produce any change in lung blood-volume. Using the method described by Daly [1928], the lung blood-volume was measured in two experiments, and no change in this value occurred during severe bronchoconstriction due to  $\text{NH}_3$  inhalation.

In an attempt to elucidate the mechanism of the bronchoconstrictor action of ammonia, further studies were made on lungs after treatment

followed by a marked loss of sensitivity to the same agent. The effect was such that when a maximal dose had already been administered, a further inhalation of phosgene either had no effect or produced a reduction in tidal air of less than 10 per cent. This may in part have been due to the fact that there was little or no recovery from the reduction in tidal air which was produced by the initial gassing. It was possible, however, that the excitable tissue, either nerve or muscle, on which the gas acted became insensitive, due to damage arising from phosgene itself.

The loss in sensitivity which occurred after one dose of phosgene, made it difficult to analyse the cause of the response. The bronchoconstriction might have been due to a direct action of phosgene on the bronchial muscle, or to an indirect action by stimulation of intrapulmonary nerve tissue. On the second hypothesis the effect should have been prevented by atropine, but tests with this drug, and nicotine, which gave similar results, were not wholly conclusive. Thus in three lungs which had been atropinised (blood concentration 1 in 100,000 to 1 in 10,000), and in two which had been treated with nicotine (50 mg or more), doses of 20–30 mg of phosgene administered so as to ensure maximal effectiveness produced a reduction in tidal air of 7 to 10 per cent. These responses were  $\frac{1}{2}$  to  $\frac{1}{3}$  of the response of the least reactive of the control series of lungs when tested in a similar manner. This gave some support to the view that the bronchoconstrictor effect of phosgene is in fact mediated by excitation of intrapulmonary post-ganglionic neurones. Nevertheless, in the absence of more satisfactory controls, the possible inhibitory actions of nicotine and atropine could not be regarded as proven. For this reason it seemed more profitable to turn our attention to a study of the action of ammonia.

(b) *Ammonia*—The inhalation of ammonia also caused marked bronchoconstriction in isolated dog lungs. In 10 preparations, additions of 5 to 20 c.c. of ammonia-air mixture produced reductions in tidal air ranging from 5 to 98 per cent. This variation was to a certain extent due to differences in the sensitivity of the preparations, but in lungs of a given sensitivity a correlation between dose and response was found, and the curve in fig. 7 illustrates one such experiment. The inset in fig. 7 is from the same experiment and shows the reduction in tidal air effected by a dose of 20 c.c. of vapour, as well as the first stages of recovery which followed.

The action of ammonia on the bronchi differed from the more immediate effects of phosgene in four respects. First there was a pronounced latent period, varying inversely with the dosage of 0.25 to 6 minutes. Secondly, recovery was a more noticeable feature of the response. Thirdly, the response could be repeated at intervals without a serious diminution in sensitivity. Finally we observed that the response might be diphasic, for slight bronchodilatation sometimes



TABLE III

Series	Number of observations	Number of individuals	P C reduction in tidal air Average response (range of response)	Standard deviation	Dosage range with air ammonia mixture, cc/vapour	Latent period in minutes	Number showing some recovery
No premedication Range	13	9	26 (0-43)	18	9.0-0.9	1-6'	6/10 (3-21 min)
Eserine only Range	20	7	65 (15-100)	29	20-3.5	1-3'	17/20 (5-20 min)
Eserine + Atropine Range	11	3	29 (4-70)	25	14-3.5	1-3'	6/7 (4-9 min)
Eserine + Nicotine Range	12	6	58 (0-05)	30	10-1	15'-3½'	9/12 (1½-19 min)
Eserine + Atropine + Nicotine Range	15	5	26 (14-60)	21	20-6	15'-4'	—

The times in the final column give the number of minutes which elapsed between time of gassing and onset of some recovery

with (a) eserine, (b) atropine, (c) nicotine, (d) anthisan given either singly or in combination. The results are briefly summarised.

Eserine greatly potentiated the bronchoconstrictor action of ammonia, while, if a diphasic response were present, it became more pronounced. Fig. 8 shows a typical experiment in which eserine was given first to the right lung, and then two and a half hours later to the left lung. A striking potentiation is shown in this experiment (a) by the contrast in response to ammonia between the eserinized and non-eserinized lung with equal doses of vapour, and (b) the increase in response to ammonia of the left lung after the administration of eserine to it as well. The same experiment shows the effect of atropine. This drug reduced the response to ammonia in eserinized lungs to an extent such that eserinized, atropinized lungs reacted to ammonia in the same way as the untreated controls. It must be emphasised that atropine did not diminish the effects obtained in uneserinized lungs. Thus in five tests on atropinized lungs, including the one illustrated in fig. 9, the tidal air reduction ranges from 6 to 79 per cent (mean 29 per cent, doses of ammonia 4 to 14 c.c.). Statistically there was no significant difference between this range of response and that obtained in the untreated controls with comparable doses of ammonia. These results refer to atropine given by either the pulmonary or the bronchial vascular route. Had all the tests been confined to sub-threshold doses of ammonia given in the presence of eserine, it would have appeared that ammonia bronchoconstriction in the isolated lung was dependent upon the release of acetylcholine, since with such doses the response was only observed in the presence of eserine. With higher doses of ammonia, however, constriction occurred without eserine. This constriction was unaffected by atropine, which indicated that another mechanism, in addition to the cholinergic one, was probably involved.

The effect of nicotine alone, and in combination with other drugs (atropine, eserine and anthisan), was tested in 18 observations. The main findings are summarised in Table III (see also fig. 8). Nicotine did not appear to reduce the response except possibly under one condition, namely, when the lungs had been given eserine and atropine already, but its action was certainly not comparable with that of atropine. Anthisan may also have reduced the bronchoconstrictor action of ammonia, but only slightly, even though the normal bronchoconstrictor action of histamine was prevented by this drug. There does not seem to be any question, therefore, that the response could have been dependent upon the liberation of histamine-like substances. No single one of the drugs mentioned, nor any combination of the three, could wholly suppress the response to doses of ammonia of more than threshold values.

In the first place, the potentiation of the ammonia response is of the same order as the potentiation of bronchoconstriction by acetylcholine, secondly, it persists long after (more than two hours) the bronchoconstriction due to eserine itself has worn off. Finally, it has been found (see fig 10) that potentiation is present while no potentiation of histamine bronchoconstriction is demonstrable, a point which Feldberg (personal communication) has said he regards as decisive.

From the foregoing it will be clear that we have no reason to implicate a release of histamine as the sole cause of the bronchoconstrictor action of ammonia vapour. In the case of the phosgene experiments, measurements of the blood and plasma histamine before and after poisoning of isolated perfused dog lungs were made by Dr J Dekanski in four experiments. He found that there were no alterations in the histamine content of the blood as the result of gassing, although the usual bronchoconstriction was observed.

A comparison of the bronchomotor actions of phosgene and ammonia has already been made. They have the common property of being able to induce bronchoconstriction by local action on the lung, as distinct from any effects produced through the extrinsic reflexes which may be observed in intact animals. So far as our evidence goes, it suggests that the two gases produce bronchomotor responses through similar mechanisms, but the tissue damage which inevitably results from phosgene poisoning has made it impossible to carry the comparison any further.

### SUMMARY

1 The reflex responses to inhaled phosgene or ammonia vapour have been observed in tracheotomised dogs, cats and rabbits, and the most important findings are

- (a) Ammonia and phosgene in high concentrations produce similar initial reactions, which are characteristic of each species and are mainly mediated by the vagi
- (b) The dog appears to possess an extra-vagal afferent system sensitive to such irritants, this system either being absent or much less sensitive in the cat and rabbit
- (c) Bronchoconstriction is apparently induced by both gases in the absence of the central connexions of the vagus

2 From studies on the isolated blood-perfused lungs of dogs, it is found that

- (a) Bronchoconstriction is produced by inhalation of either phosgene or ammonia
- (b) The bronchoconstrictor action of ammonia is specifically potentiated by eserine, an effect which can be reversed by atropine

## DISCUSSION

Two points concerning these results should be emphasised. In the first place, it has been found that ammonia can produce bronchoconstriction under conditions which exclude the participation of the extrinsic nervous system, a fact which does not seem to have been observed earlier. It is analogous to the findings of Laqueur and Magnus [1921] on cats' lungs, and Daly, Eggleton, Elsdon and Hebb [1946] on isolated dogs' lungs, that phosgene has a bronchoconstrictor action under these conditions, and is a factor which should be borne in mind in assessing the whole animal's responses to these gases in concentrations we have employed. The second point concerns the potentiation of the response by eserine. Atropine has the power of nullifying any potentiation due to eserine, but cannot suppress the effect of greater than threshold doses of ammonia. It would therefore appear that the response depends not only on the activation of nervous tissue with a consequent release of acetylcholine, but also upon a stimulation of the muscle itself independently of its innervation.

There are certain considerations which indicate that ammonia might well have an action both on nerve endings and on muscle. It has been found in experiments recently carried out in this laboratory that ammonium chloride has an action similar to, though weaker than, that of potassium chloride in stimulating the perfused superior cervical ganglion of the cat, a result which is analogous to that found by Hermann [1923 *a, b, c*] on the adrenal medulla. Potassium chloride itself has been shown (1) to release acetylcholine from preganglionic nerve endings [Brown and Feldberg, 1936], and (2) to stimulate the post-ganglionic nerve-cells independently of the release of acetylcholine [Brown *et al*, 1936]. Further, in experiments on normal and denervated striated muscle [Altamirana *et al*, 1948], it has been found that the action of potassium ions is potentiated by eserine on the first but not on the second. There is thus good reason to assign a double action to potassium chloride on normally innervated ganglia or striated muscle. If, as it seems probable, the action of ammonia and potassium ions is similar, then the same explanation can be applied to the present experiments. It should be noted here that the absence of any definite effect of nicotine on the response suggests that ammonia stimulation of nerve tissue, if it occurs, is at a point peripheral to the synaptic junctions of the intrapulmonary ganglia.

Ambache's conclusion, however, that acetylcholine is liberated in the gut by barium chloride and other agents because their action on this tissue is potentiated by eserine, has been criticised by Feldberg on the grounds that the potentiation in such circumstances is not specific. Without entering into the merits of Feldberg's arguments, it may be noted that these criticisms do not apply in the present experiments.



- (c) The stimulating action of greater-than-threshold doses of ammonia in non-eserimized lungs is not affected by atropine, nicotine or anthisan

3 From the evidence obtained in the second group of experiments, it is suggested that ammonia may have a direct stimulating action on the bronchial smooth muscle in addition to any action mediated by peripheral nervous structures and the release of acetylcholine

#### ACKNOWLEDGMENTS

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## W H. NEWTON

THE members of the Editorial Board record with profound regret the death of their Chairman, Professor W H NEWTON, on December 20, 1949. When he came to Edinburgh in 1948 as Professor of Physiology, he was faced with formidable problems as the head of a large Department at a time when the curriculum was being altered and structural changes in the building had become essential. He nevertheless undertook with enthusiasm the added burden of editing this Journal, which seemed to be assured of continued development under his guidance. His untimely death has been a severe blow to the Editorial Board of the *Quarterly Journal of Experimental Physiology*, as it has been to his colleagues in many other fields of work.



THE USE OF INULIN CLEARANCE AS A MEASURE OF  
GLOMERULAR FILTRATION By M H FERGUSON, O  
OLBRICH, J S ROBSON and C P STEWART From the Clinical  
Laboratory, Royal Infirmary, Edinburgh

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INTRODUCTION

THE proposition that inulin is excreted in the urine solely by glomerular filtration, and that therefore its renal clearance is a valid measure of the filtration rate in man, was first advanced by Shannon and Smith [1935]. Apart from evidence which relates to species other than man, the claim is based upon three types of observation [Miller, Alving and Rubin, 1940]: (1) when tubular function is depressed by phloridzin, or when the tubular cells are saturated with glucose, ascorbic acid, etc., the clearances of a number of compounds approach the value of the inulin clearance [Smith, 1937], (2) the inulin clearance remains constant when the plasma concentration of inulin is varied over a wide range [Shannon and Smith, 1935], (3) the endogenous creatinine clearance is equal to the inulin clearance in man [Miller and Winkler, 1938, Brod and Sirota, 1948]. Of these, the *sine qua non* for the acceptance of the validity of inulin clearance as a measure of the rate of glomerular filtration is the absence of any systematic variation in the magnitude of the clearance as a result of changes in the plasma concentration of inulin within wide limits.

Shannon and Smith [1935] claimed to establish the constancy of the renal clearance of inulin between plasma inulin levels of 50 and 400 mg/100 ml. Since extrapolation of the straight line which they constructed, relating the renal excretory rate of inulin and the corresponding plasma levels, passed through the point of origin, they concluded that the independence of inulin clearance and plasma levels still persisted at plasma levels below 50 mg/100 ml, and that the slope of the regression line represented the rate of glomerular filtration. Shannon and Smith did not further attempt to dissociate errors in observation of renal clearance from systematic variations that might exist in the clearance of inulin at varying plasma levels. The method they employed in constructing the regression line relating the renal excretory rate to the plasma levels of inulin was, apparently, one of visual approximation.

which, without offence to their data, permitted the lines to be drawn through the point of origin. Any small deviation from the point of origin that might have been inherent in their figures would thus be obscured.

Shannon and Smith [1935], using the relatively crude analytical methods of those days, were unable to measure the renal clearance of inulin at low plasma levels. Miller, Alving and Rubin [1940], however, with improved methods at their disposal, attempted to define the relationship between the plasma inulin and renal excretory rate at levels between 85 and 5 mg per 100 ml plasma, pointing out the inadequacies of the analysis of Shannon and Smith. The experiments of Miller, Alving and Rubin were carried out both with plasma levels falling after a single intravenous injection of inulin, and with levels kept constant by continuous intravenous infusion. They claimed to confirm the findings of Shannon and Smith [1935]. These results, the importance of which was emphasised by Shannon [1942], have led to wide acceptance of the statement that the slope of the line relating the excretory rate of inulin and the plasma level is a measure of filtration at all plasma levels. Inherent in the use of the concept that the clearance of inulin is constant, and is equal to  $\frac{uv}{P}$ , is the assumption that the regression line relating the two variables passes through the point of intersection of the co-ordinates.

Recently, the authors [Robson, Ferguson, Olbrich and Stewart, 1949] devised a method for the estimation of the average renal clearance of inulin from the slope of the falling plasma inulin concentrations plotted against time. In the course of this work, the clearance was measured during a number of successive periods, and it was observed (though it was not commented on in the publication) that the values for clearance tended to fall steadily as the plasma level fell. The present paper consists of an analysis of these data along with other data from specially designed experiments.

## EXPERIMENTAL

*Source of Data*—Fifteen subjects in whom the renal clearance of inulin had been determined were selected for this analysis. The sole criterion for inclusion in the series was that clearance had been determined for a minimum of five consecutive clearance periods following a single intravenous injection of inulin, the first period being at least twenty minutes after the injection.

In the majority of these subjects, clearances were estimated for seven periods. The clearance periods were of 10 to 20 minutes' duration, and during the experiments the plasma level of inulin ranged from 64.0 to 4.8 mg/100 ml. Four of the subjects were normal healthy

individuals, the remaining eleven were elderly patients suffering from varying degrees of essential hypertension, but who otherwise had no complaints

*Experimental Procedure*—Data on some of the subjects has already been published [Robson, *et al*, 1949], and the preparation of the other subjects was as previously reported. All subjects fasted overnight and were recumbent during the experiment, no breakfast was given, and a free flow of urine was established by allowing water to be drunk before the experiment. Inulin, dissolved in saline, was given as a single intravenous injection in amounts of approximately 120 mg/kg body-weight. In each case the injection was completed in about five minutes. Venous blood was removed at intervals of ten to twenty minutes for two to three hours after injection, urine was collected by catheter over periods of ten to twenty minutes for a similar length of time, the bladder being washed out with 20 ml saline after each collection. The exact time was noted for all operations. Inulin was determined in all plasma and urine samples by Cole's unpublished modification of Steinitz's method [1938].

*Calculation of Clearance*—For each period the renal clearance of inulin was determined by dividing the mean rate of inulin excretion per minute by the estimated mean plasma level of inulin for the period. The latter was read from a smooth curve drawn through the plasma inulin concentrations plotted against time. The number of estimations of plasma inulin during the experiment permitted a good curve to be drawn easily. The concentration selected was one occurring  $2\frac{1}{2}$  minutes before the mid-point of the clearance period.

*Statistical Analysis of Data*—On the basis of the claims of Shannon and Smith [1935], and Miller, Alving and Rubin [1940], straight lines were fitted to the paired values of the rate of renal excretion of inulin ( $uv$ ) and the mean plasma levels ( $P$ ) obtained from the smooth curve of plasma levels plotted against time, and selected  $2\frac{1}{2}$  minutes before the middle of each period. The method of least squares was employed. The formula used is one where it is assumed that the weight of  $P$ /weight of  $uv = \alpha$ ,  $\pm e$  where the independent variable (here  $P$ ) is free from error compared with  $uv$ . Although this is not completely true, it is clear that errors in  $P$  are certainly small in relation to the errors in  $uv$ , since the values for  $P$  are taken from a smoothed curve through seven to nine determinations of plasma inulin which are subject solely to errors of chemical analysis (S D 2.5 per cent). The estimations of  $uv$ , however, are subject to errors not only of chemical analysis, but also in the estimation of the volume of urine voided in unit time—a measurement based upon the collection of urine by catheter over periods of 10 to 20 minutes. This procedure is the largest technical source of error in clearance determinations.

The suggestion that the lag-time between filtration and the arrival

TABLE I—VALUES FOR THE RATE OF RENAL EXCRETION OF INULIN AND THE CORRESPONDING PLASMA INULIN CONCENTRATION READ 2½ MINUTES BEFORE THE MID POINT FOR EACH CLEARANCE PERIOD. VALUES FOR CLEARANCE ARE CALCULATED FROM  $C=uv/P$ . AGE, SEX AND BLOOD PRESSURE OF THE SUBJECTS ARE GIVEN

Subject	Sex	Age, years	B P, mm Hg	$uv$ inulin, mg/min	$P$ inulin, mg/100 ml plasma	$C=uv/P$ ml/min plasma
1 W L	M	25	145/95	55.1	30.5	180.7
				43.0	23.5	183.0
				29.9	18.7	159.9
				13.1	10.5	124.6
				11.1	9.1	121.5
				9.6	8.0	120.4
				7.9	7.2	109.7
2 W S	M	28	130/80	64.0	37.0	173.0
				40.1	26.5	151.3
				30.9	21.0	147.1
				16.4	13.7	119.7
				13.5	12.0	112.5
				12.0	10.6	113.2
				11.1	9.7	114.4
3 M.R	F	25	120/70	56.6	54.5	103.0
				35.5	37.7	94.2
				29.2	30.0	97.3
				21.8	25.3	85.4
				18.0	22.0	81.8
4 G M	M	70	190/110	56.8	56.0	101.4
				35.4	41.0	86.3
				26.6	32.5	81.8
				21.0	27.0	77.8
				17.2	23.0	74.8
				14.5	20.0	72.5
				13.3	18.5	71.9
5 W H	M	44	140/90	57.1	57.0	100.2
				39.3	39.0	100.8
				26.3	28.5	92.3
				21.0	22.6	92.9
				16.0	19.4	82.4
				14.5	16.8	86.3
				11.0	14.8	74.3
6 M L	F	83	200/110	32.8	30.5	83.0
				24.3	31.0	78.4
				18.0	26.8	67.2
				17.5	23.0	76.1
				15.0	20.2	74.3
				10.2	13.0	56.7
				9.2	16.5	55.8
7 E T	F	82	190/100	39.0	43.5	89.7
				26.0	31.0	83.9
				17.9	24.5	73.1
				10.6	16.0	66.3
				9.5	14.6	65.1
				7.8	13.5	57.8
				4.8	12.2	39.3

TABLE I—continued

Subject	Sex	Age, years	B P, mm Hg	uv inulin, mg /min	P inulin, mg /100 ml plasma	C=uv/P ml /min plasma
8 B J	F	62	210/130	36.7	45.0	81.6
				22.1	32.0	69.1
				17.9	25.0	71.6
				14.0	21.5	65.1
				12.2	19.0	64.2
				10.8	17.0	63.5
				8.7	16.0	54.4
9 R.D	F	75	230/120	37.8	48.4	78.1
				26.0	36.8	71.0
				18.9	28.2	67.0
				13.9	20.0	69.5
				11.1	17.6	63.1
				9.5	15.6	60.9
10 M.M.	F	72	250/140	37.6	47.0	80.0
				26.5	34.0	77.9
				12.7	16.0	79.4
				10.2	13.7	74.5
				8.6	11.8	72.9
11 H.L.	M	75	200/130	33.5	47.0	71.3
				29.1	37.0	78.7
				23.1	30.5	75.7
				18.6	24.8	75.0
				14.4	21.5	67.0
				13.5	18.3	73.8
12 J.O.	M.	75	230/115	28.9	47.2	61.2
				20.5	37.8	54.2
				18.3	31.5	58.1
				16.0	26.8	59.7
				11.2	23.5	47.7
				8.8	20.8	42.3
13 C.B.	F	68	220/125	7.4	17.8	41.4
				35.9	62.0	57.9
				24.3	42.0	57.9
				20.0	31.5	63.5
				13.9	25.5	54.5
				13.5	22.5	60.0
14 B.D.	F	65	160/100	11.2	20.5	54.6
				9.3	18.8	49.5
				25.5	46.0	55.4
				19.1	33.0	57.9
				13.2	26.0	50.8
				10.3	21.5	47.9
15 J.P.	F	70	220/120	9.8	18.4	53.3
				8.2	16.2	50.6
				6.6	14.2	46.5
				29.0	53.0	54.7
				20.0	40.4	49.5
				14.4	33.0	43.7
				13.9	27.8	50.0
				10.9	24.0	45.4
				9.4	21.0	44.8
				9.4	18.6	50.5

of the modified filtrate in the bladder is 6 minutes has been made by Brun, Hilden and Raaschou [1949], though the original estimate of  $2\frac{1}{2}$  minutes made by Smith, Goldring and Chasis [1938] appears well substantiated. In view of this disparity of opinion, however, regression equations of  $uv$  upon  $P$  plotted against time were also calculated with values of  $P$  read 6 minutes prior to the mid-point of the period.

The significance of any deviation of the regression lines from the point of intersection of the co-ordinates was estimated from an analysis of variance about the regression and the statistic  $t$ . Values of  $P$  (probability) were then read from Fisher's table of  $t$  for  $n-2$  degrees of freedom [Fisher, 1941].

## RESULTS

Table I presents the results of the renal clearance of inulin for the individual clearance periods for the fifteen subjects. The rates of secretion of inulin and the corresponding values for plasma inulin concentration derived from the plasma inulin-time curve,  $2\frac{1}{2}$  minutes before the mid-point of the period for which the values for clearance were calculated, are shown. The ages in years and the blood-pressures of the subjects are also given. With the exception of cases M M, C B, and H L, all subjects exhibit a steady fall in the renal clearance following the intravenous injection, the value of the last determined inulin clearance being from 16 to 56 per cent lower than the value for the first clearance period in the same subject.

The nature of this phenomenon is, however, best observed in Tables II and III, in which the equations of the regression lines of  $uv$  upon  $P$

TABLE II — LINEAR REGRESSION LINES,  $uv = aP - b$ , FITTED TO EXPERIMENTAL DATA

Subject	$uv = aP - b$	Variance	$n - 2$	Probability
1 W L	$uv = 2.073 P - 7.579$	1.478	5	< 0.001
2 W S	$uv = 1.937 P - 9.245$	2.234	5	< 0.001
3 M.R	$uv = 1.175 P - 7.653$	1.627	3	< 0.050
4 G.M	$uv = 1.144 P - 9.215$	2.770	5	< 0.010
5 W.H	$uv = 1.089 P - 4.349$	0.749	5	< 0.010
6 M.L	$uv = 0.999 P - 6.855$	1.699	5	0.010
7 E.T	$uv = 1.050 P - 6.796$	0.800	5	< 0.001
8 B.J	$uv = 0.924 P - 5.662$	0.982	5	< 0.010
9 R.D	$uv = 0.841 P - 3.783$	0.728	4	< 0.020
10 M.M	$uv = 0.813 P - 0.789$	0.205	3	< 0.200
11 H.L	$uv = 0.781 P - 1.165$	1.986	4	< 0.700
12 J.O	$uv = 0.772 P - 5.319$	1.444	5	< 0.020
13 C.B	$uv = 0.599 P - 0.785$	1.022	5	< 0.500
14 B.D	$uv = 0.600 P - 1.785$	0.512	5	0.050
15 J.P	$uv = 0.571 P - 2.466$	1.270	6	0.050

The values for  $P$  are taken  $2\frac{1}{2}$  minutes before the mid point of the corresponding urine collection period.  $uv$  is expressed in mg. inulin per minute.  $P$  in mg. inulin per 100 ml. plasma.



TABLE III — LINEAR REGRESSION LINES,  $uv = aP - b$ , FITTED TO EXPERIMENTAL DATA

Subject	$uv = aP - b$	Variance	$n - 2$	Probability
1 WL	$uv = 1.886 P - 6.258$	1.528	5	< 0.010
2 WS	$uv = 1.772 P - 7.871$	1.423	5	< 0.001
3 MR	$uv = 1.044 P - 4.980$	1.250	3	0.050
4 GM	$uv = 1.022 P - 6.917$	1.492	5	< 0.010
5 WH	$uv = 0.946 P - 2.199$	0.817	5	< 0.050
6 ML	$uv = 0.926 P - 6.036$	1.632	5	< 0.020
7 ET	$uv = 0.884 P - 4.355$	1.473	5	< 0.010
8 BJ	$uv = 0.825 P - 4.035$	0.439	5	< 0.010
9 RD	$uv = 0.754 P - 2.340$	0.213	4	< 0.001
10 MM	$uv = 0.736 P - 0.626$	1.258	3	< 0.700
11 HL	$uv = 0.732 P - 0.481$	1.991	4	0.800
12 JO	$uv = 0.672 P - 4.953$	0.853	5	0.020
13 CB	$uv = 0.581 P - 0.789$	1.531	4	< 0.700
14 BD	$uv = 0.545 P - 1.047$	0.516	5	0.200
15 JP	$uv = 0.521 P - 1.654$	1.222	6	< 0.200

The values for  $P$  are taken 6 minutes before the mid point of the corresponding urine collection periods. Units for  $uv$  and  $P$  are as in Table II.

are given, and in figs. 1 and 2, which illustrate the lines given in Tables II and III respectively. Table II includes the equations employing

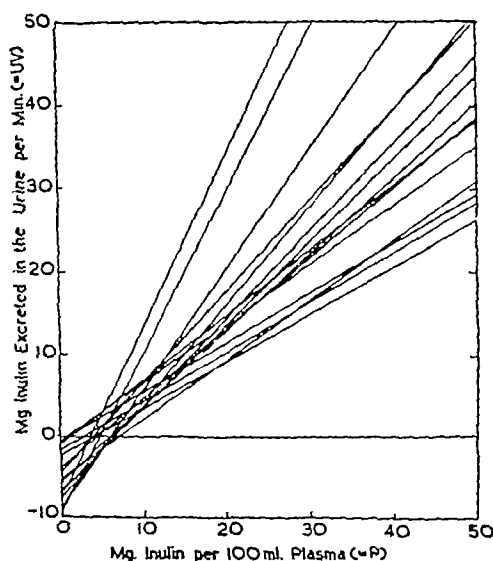


FIG. 1 — Regression lines of  $uv$  upon  $P$  for fifteen subjects drawn from equations given in Table II. The mean plasma inulin concentration ( $P$ ) for each clearance period was selected  $2\frac{1}{2}$  minutes before the mid point.

values of  $P$  derived  $2\frac{1}{2}$  minutes from the mid-point of the clearance period. Table III presents the equations of the lines recalculated employing values of  $P$  derived from the plasma inulin-time curve.

6 minutes prior to the mid-point of the period. The lines are given in the form  $uv = aP - b$ , where  $a$  and  $b$  are the constants calculated by the method of least squares. That the lines do not pass through the point of origin is seen from the magnitude of the constant  $b$  for the individual subjects. This constant has a value varying from 0.789 to 9.245 when  $P$  is taken  $2\frac{1}{2}$  minutes before the mid-point of the period, and a value

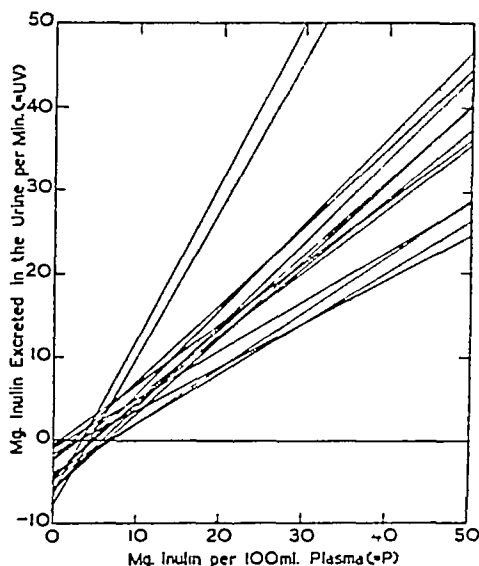


FIG. 2—Regression lines of  $uv$  upon  $P$  for fifteen subjects drawn from equations given in Table III. The mean plasma inulin concentration ( $P$ ) for each clearance period was selected  $2\frac{1}{2}$  minutes before the mid point.

varying from 0.481 to 7.871 when  $P$  is taken 6 minutes before the mid-point of the period. The tables include the variances about the regression and the significance of the deviations of the constant  $b$  from the point of origin. Employing values of  $P$  taken  $2\frac{1}{2}$  minutes before the mid-point, twelve of the fifteen subjects show a significant ( $P < 0.05$ ) deviation of their regression lines from the point of origin, and the  $t$  test shows that, for the whole group, the deviation is highly significant ( $t = 6.29$ ). When the values of  $P$  are taken 6 minutes before the mid-point of the clearance period, the deviation is significant in ten of the fifteen subjects and highly so for the whole group, since  $t = 5.04$ , which indicates  $P < 0.001$ . In all subjects the value of  $b$  is positive.

#### DISCUSSION OF RESULTS I

It is clear from the results in general that, in the fifteen subjects studied, the regression of  $uv$  upon  $P$  does not pass through the point of

origin This is so irrespective of whether values of  $P$  are taken  $2\frac{1}{2}$  or 6 minutes before the mid-point of the clearance period It may obviously be presumed that when there is no inulin in the plasma there is none in the urine, and therefore the results imply that the regression line is truly linear but does not pass through the origin, or that it is curvilinear in at least the part where  $P$  is small In either case it follows that the rate of excretion of inulin is not proportional to the plasma concentration selected as representing the mean concentration of inulin for the clearance period

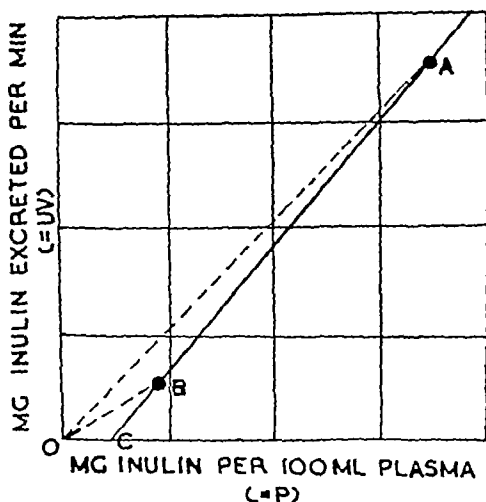


FIG 3

Irrespective of any interpretation imposed upon this result, it is possible to explain the fall in values of the renal clearance of inulin obtained by using the formula,  $\text{Clearance} = \frac{uv}{P}$  This is readily seen from inspection of fig 3 (diagrammatic), in which two points, A and B, representing two paired observations of  $uv$  and  $P$  at different values of plasma inulin concentration ( $P$ ), are plotted in such a way that the extrapolation of the line AB intersects the positive abscissa at C, and if continued would intersect the negative ordinate The function  $\frac{uv}{P}$  represents the slopes of the line AO or BO according to the value of  $P$  used in the expression It is clear that the slope of any line estimated by the function  $\frac{uv}{P}$  will progressively diminish with smaller values of  $P$  and will correspond to the slope of ABC only when  $P$  is infinite

In view of these findings, it is important to decide whether the relationship between  $uv$  and  $P$  following a single intravenous injection

provides a true expression of the relation between the two variables, or whether the relation which is found is due to the method of administration and the dynamics of distribution throughout the body, including the kidney, rather than to the mechanism of excretion

The original experiments of Shannon and Smith [1935] were performed on subjects who received a large intravenous injection of inulin over a period of about 30 minutes, and the clearances were calculated from data obtained when the plasma concentration of inulin was subsequently falling rapidly, using values of  $P$  determined at the mid-point of the clearance period. That the data from these experiments could be held to indicate constancy of the value of  $\frac{uv}{P}$  appears to be due

to (a) the high plasma inulin levels, (b) the use of direct plasma inulin determinations by a relatively crude method rather than values from a smooth curve, and (c) the visual fitting of the regression line. All of these factors tend to obscure the small deviation from constancy which we have regularly found. On the other hand, Miller, Alving and Rubin [1940], working with a plasma inulin range similar to our own, using an accurate method of determining inulin, and taking values for  $P$  corresponding to the mid-point of each period from a smoothed curve, did not find the inulin clearance to fall regularly with falling plasma inulin, their results being equivocal, they found a steady fall in one case (A W), a steady rise in another (L B), and in three cases, in which the variance about the regression line which we have calculated from their published data was large, there was no definite trend in either direction. It must be confessed that we can offer no satisfactory explanation of this discrepancy, especially as the use of  $P$  values taken at the mid-point of the clearance periods would exaggerate the tendency we have found for clearances to fall with  $P$ .

At this stage it seemed just possible that a single injection of inulin, by producing different concentrations in the arterial and venous blood, might itself be the cause of the apparently varying relationship between the plasma concentration of inulin ( $P$ ) and the amount excreted per minute ( $uv$ ). The method of intravenous infusion ensures that the arterial and venous concentrations of inulin are equal [Brun, Hilden, Raaschou, 1949], since the rate of infusion is normally adjusted to approximate the rate of excretion. When the single injection technique is employed this no longer holds, and it is possible that the sampling of venous blood may introduce an error into the calculation of clearance. Since the kidney receives arterial blood, the magnitude of any error so introduced would be represented by the arterio-venous difference in inulin concentration. In the following section the possible magnitude of this error is investigated and discussed.

## INVESTIGATION OF THE ARTERIO-VEINUS DIFFERENCE

The arterio-venous difference of plasma inulin concentrations was investigated in five subjects following a single intravenous injection of inulin. All subjects were elderly and suffering from varying degrees of essential hypertension. In addition, case W P had undergone a supra-pubic cystostomy for benign enlargement of the prostate.

*Experimental*—The subjects were prepared as described in the preceding section. A single intravenous injection of inulin dissolved in saline was given in amounts of approximately 120 mg/kg body-weight. Starting about 20 minutes after injection, arterial and venous blood samples were obtained at intervals of about 20 minutes. Five to seven pairs of such samples were obtained for each case. Venous blood was obtained from a vein in the cubital fossa, and arterial blood was withdrawn from the femoral artery just below the inguinal ligament. An indwelling cannula was not used. In addition, two samples of urine were obtained approximately 30 and 100 minutes after injection. This permitted an estimation of the average renal clearance of inulin to be performed, using the method of Robson *et al* [1949]. All operations were accurately timed, and the inulin concentration of all samples was determined by the method already indicated.

*Results*—Since venous and arterial blood samples were obtained at slightly different times in the same subject, direct comparison of the two concentrations was not possible. Accordingly, all venous concentrations were plotted against time, and a smooth curve constructed through the points by visual approximation. The arterial concentrations were then plotted on the same graph, and were compared with the venous plasma concentrations read from the smooth curve at the times of the arterial estimations. In this way five to seven estimations of arterio-venous differences were made in each of the five subjects.

The results are shown in fig 4, and the arterio-venous differences calculated at the times of the arterial estimations are given in Table IV. From the figures it is seen that there is almost complete superimposition of the arterial concentrations upon the curve drawn through the venous determinations.

The average arterio-venous difference for each individual varies from +1 per cent to -3 per cent. Although in one or two instances the difference is as great as 9 per cent, the claim that the arterial concentration of inulin is at least proportional and, indeed, very nearly equal to the venous level throughout the duration of the experiment, following a single intravenous injection, may be made from an inspection of fig 4 alone. The individual estimations of the differences between arterial and venous blood suffer from the fact that the errors of analysis in two independent estimations may be additive, though the effect of this is minimised by the smoothing of one set of results. Estimates

#### TABLE IV—THE CONCENTRATION OF INULIN IN ARTERIAL AND VENOUS PLASMA

Values for the average clearance of Inulin by the authors' method [1949] are given for the subjects. Values for Inulin Concentration in Venous Plasma were obtained from a smooth curve drawn through Venous Plasma Inulin Concentration plotted against time at the time of obtaining arterial blood. Arterial Plasma Concentrations given are those determined directly from samples. The difference is expressed as percentage of the Venous Concentration. Time,  $t$ , indicates the time in minutes following injection

Subject	Sex	Age, years	B P, mm Hg	Inulin, given mg/kg	$C_{in}$	Time, $t$	$P$ Venous, smoothL	$P$ Arterial	$A - V$ difference, $\Delta$	% $\Delta$ of venous	
W F	M	68	205/95	118	103	19.5	58.0	55.5	-3.1	-5	
						44	38.8	37.5	-1.3	-3	
						66	28.0	27.7	-0.3	-1	
						87	21.4	20.7	-0.7	-3	
						110	16.0	15.8	-0.2	-1	
						134.5	12.7	12.5	-0.2	-2	
Average percentage difference											-3
C M	M	71	170/100	116	91	20	64.5	63.0	-1.5	-2	
						41.5	44.5	47.5	3.0	+6	
						61.5	35.0	31.5	-3.5	-11	
						93	26.0	25.7	-0.3	-1	
						114	22.0	21.0	-1.0	-5	
						133	19.0	17.7	-1.3	-7	
160	16.3	15.5	-0.8	-5							
Average percentage difference											-3

J C	M	69	186/86	137	50	19	59.5	59.5	0	0	-3
						35	45.5	45.5	0	0	
						69	32.1	32.4	+0.3	+0.9	
						91	26.7	23.0	-2.8	-8.0	
						113	22.8	20.8	-2.0	-9.0	
						Average percentage difference					
J S	M	77	195/100	107	40	23.5	73.0	78.3	+5.3	+7	
						42.5	57.6	54.7	-2.9	-5	
						65	46.2	46.2	0	0	
						92	39.4	39.9	+0.5	+1	
						123.5	33.0	32.7	-1.2	-4	
						Average percentage difference					-0.2
W P	M	76	240/100	97	21	16	57.0	50.5	+2.5	+4	
						31.5	46.0	46.7	+0.7	+1	
						51.5	39.7	39.7	0	0	
						69	36.9	38.3	+1.4	+4	
						91	34.2	33.0	-1.2	-4	
						115	32.0	32.9	+0.9	+3	
						139	29.3	29.3	0	0	
						Average percentage difference					+1

of the renal clearance of inulin in these subjects are also given in Table IV

*Discussion*—The discussion of these results will be confined to the relation they bear to the phenomenon of the systematic decline in the magnitude of the renal clearance of inulin following a single injection, and described in the preceding section. It is clear that any absolute difference between the concentrations of inulin in the venous plasma and in the arterial plasma will affect the value for clearance by the magnitude of that difference. The slopes of the regression lines of  $uv$  upon  $P$  will

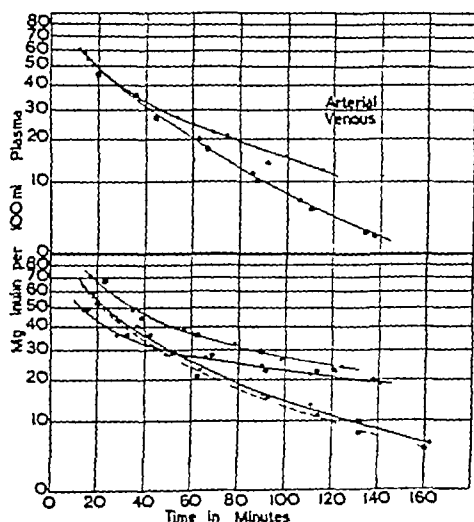


Fig. 4—The arterial and venous plasma inulin concentrations plotted semi logarithmically with time following a single intravenous injection in five subjects. The injection was given at time = 0. The unbroken lines were drawn by visual approximation through the individual venous plasma inulin determinations. The broken line is drawn for one subject only through the individual arterial plasma determinations.

accordingly be modified. In view of the results, the modification is slight and almost beyond the precision of the estimation. Under these circumstances, the points at which the regression lines cut the ordinate in the fifteen subjects previously described will not be affected, since these points correspond to negative values of  $u$  when  $P = 0$ . The intersection of the abscissæ by the regression lines will be proportionately modified, but this is not relevant to the present problem. It may be concluded, therefore, that differences between the venous and arterial concentrations of inulin, following the single injection of inulin, do not explain the fall observed in the clearance of inulin with falling plasma concentrations.



## OBSERVATION ON THE RENAL CLEARANCE OF INULIN EMPLOYING CONTINUOUS INFUSION

The disparity between, on the one hand, the claims of Shannon and Smith [1935], and of Miller, Alving and Rubin [1940], relating the renal excretory rate of inulin and the plasma concentration, and, on the other hand, the conclusions which are apparent from our own data, employing a single intravenous injection of inulin, suggested the need to investigate further the relationship between the two variables when inulin is given by continuous infusion. In this way the influence of the method of administration is changed, in so far as it affects the dynamics of distribution through the body. The use of the continuous infusion technique, unfortunately, makes it more difficult to interpret variations in the clearance values than when the single injection method is used. Variations in the ratio  $uv/P$  are the result of variations either of the numerator or denominator or both, and errors in  $P$  cannot be minimised by smoothing of determinations. Furthermore, the plasma concentration of inulin cannot be maintained absolutely constant by continuous infusion, especially when higher concentrations are being employed. These difficulties are to some extent overcome by considering, at each approximately constant plasma level, the average of three or four consecutive clearance determinations. If this is done, the effect of errors in collection of urine at the individual clearance periods will tend to disappear. Even so, statistical examination of the results is difficult, and is best confined to simple comparison of means of several clearance determinations, with the knowledge that the standard deviation of the determination of the renal clearance of inulin by the infusion technique has been estimated to be about 8 per cent (Smith, Goldring and Chasis [1938] found 8.9 per cent, and Olbrich, Ferguson, Robson and Stewart [unpub. data] found 7.0 per cent).

*Experimental*—Three volunteers of ages 49, 58 and 70 years respectively were used for the experiments. They suffered from varying degrees of essential hypertension, but were otherwise active and in good health. Two types of experiment were performed.

In all three subjects, following priming injections of inulin (60–80 ml 10 per cent), a continuous infusion of inulin was maintained for a period of about 90 minutes. Three or four clearances were determined during the last 60 minutes of this period, after which the infusion was stopped for a further 30 minutes. The infusion was then restarted at a slower rate for another 90 minutes, during the last 60 minutes of which three or four clearances were determined. In two of the subjects the infusion was again interrupted for 60 minutes, and again restored at a still slower rate, when after an interval of 20–30 minutes three or four clearances were determined.

The second type of experiment was performed on one of the above

subjects two weeks after the first experiment. Here the reverse procedure was adopted, consecutive clearances being determined at each of three levels of plasma inulin, which were progressively raised from lower levels. A priming injection of 10 ml 10 per cent inulin was given and the infusion commenced at a slow rate. After about 30 minutes three consecutive clearances were determined, a second priming injection was then given (20 ml 10 per cent) and the infusion was continued at a slightly increased rate. After about 30 minutes another three clearances were determined. The procedure was repeated following a third priming injection (50 ml 10 per cent) and a further three clearances were determined, the infusion being administered at a steady high rate. In all these infusions the rate was that calculated to give a constant plasma inulin level.

The inulin infusion fluid used in these experiments was as described by Goldring and Chasis [1944]. Clearance periods were of 10 to 20 minutes' duration, and venous blood samples were withdrawn  $2\frac{1}{2}$  minutes before the beginning and  $2\frac{1}{2}$  minutes before the end of each period. The mean plasma concentration of these two samples was employed in the calculation of clearance for the period. The bladder was washed out with 20 ml normal saline at the end of each period. All operations were timed, and inulin was determined in all samples by the method already indicated. The preparation of the subjects was as described in preceding sections. All subjects were recumbent during the experiment.

*Results*—The results of these experiments are given in Tables V and VI. Table V presents the data relative to subject CM, upon whom estimations of the renal clearance of inulin were determined both at diminishing levels of plasma inulin concentration, and with levels increased stepwise by repeated priming injections.

The individual plasma inulin concentrations and the values of clearance for each period are shown, along with average values at each of the six plasma levels. During diminishing levels of plasma inulin there is a definite and steady diminution in the magnitude of the inulin clearance. The average clearances at the lower levels are 9 and 12 per cent lower than at the highest level respectively. In the experiments with rising levels of plasma inulin concentration there appears a steady definite rise in the average values for inulin clearance. The two lower levels of plasma inulin provide clearances respectively 26 and 12 per cent lower than the third clearance at the highest level investigated. Furthermore, considering the two series together, the results which consist of data from 22 clearance periods performed upon one subject at six different levels of plasma inulin, provide evidence of a direct dependence of the renal clearance of inulin upon the plasma concentration. For this subject individual values of  $u_1$  are plotted against  $P$  in fig 5, which, in addition to the results of the experiments outlined

TABLE V—SUBJECT CM, 70 YEARS B P 175/100 mm Hg THE RENAL CLEARANCE OF INULIN DETERMINED AT DIFFERENT PLASMA LEVELS OF INULIN, EACH LEVEL BEING MAINTAINED APPROXIMATELY CONSTANT BY CONTINUOUS INTRAVENOUS INFUSION OF INULIN

Determinations at diminishing plasma levels		Determinations at increasing plasma levels	
Plasma inulin conc , mg /100 ml	Plasma inulin clearance, ml /minute	Plasma inulin conc , mg /100 ml	Plasma inulin clearance, ml /minute
96 0	100 7	15 5	71 7
87 7	108 3	14 3	68 2
90 0	106 5	13 7	113 1
		13 6	79 7
	Average 105 2		Average 83 2
79 0	101 8	44 3	98 5
79 0	91 0	42 3	100 6
79 0	94 9	46 1	98 3
	Average 95 9		Average 98 5
44 0	92 0	102 2	112 1
46 5	92 7	102 5	126 2
46 5	94 0	108 5	99 6
	Average 92 9	115 2	111 6
			Average 112 4

TABLE VI—THE RENAL CLEARANCE OF INULIN DETERMINED AT DIFFERENT PLASMA LEVELS OF INULIN, EACH LEVEL BEING MAINTAINED APPROXIMATELY CONSTANT BY INTRAVENOUS INFUSION OF INULIN DETERMINATIONS WERE MADE AT DIMINISHING PLASMA LEVELS

Subject W R, 49 years, B P 145/105 mm Hg		Subject J C, 58 years, B P 160/100	
Plasma inulin conc , mg /100 ml	Plasma inulin clearance, ml /minute	Plasma inulin conc , mg /100 ml	Plasma inulin clearance, ml /minute
70 8	153 1	88 0	99 6
76 1	170 4	84 7	103 5
72 3	149 4	84 0	84 6
73 2	151 1	86 2	88 4
	Average 156 0		Average 94 0
52 3	133 3	33 3	76 3
53 3	137 1	30 3	95 7
51 5	138 2	26 1	87 0
	Average 136 2	24 4	91 4
			Average 87 6
30 3	146 5		
30 0	144 0		
29 3	129 0		
	Average 139 8		

here, includes 6 other clearance periods obtained about 18 months previously, both by the single injection and the continuous infusion method. The lines drawn were constructed by the method of least squares, line B including all the points, and line A only those with  $P$  above 70 mg/100 ml.

The results obtained in the other two subjects (W R and J C) are given in Table VI. In subject W R the average clearances determined at the two lower plasma inulin levels are 13 per cent and 10 per cent

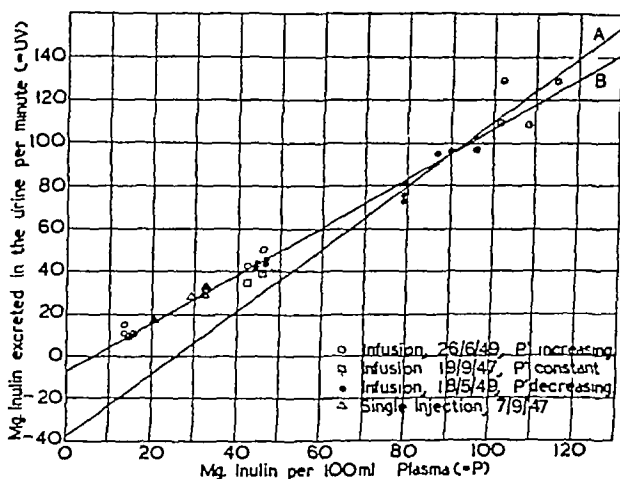


FIG. 5—Subject C M. Values for the rate of inulin excretion ( $uv$ ) plotted against values for plasma inulin concentration ( $P$ )

lower than the clearance determined at the highest level. The clearances determined at levels of about 50 mg/100 ml are, however, not very different from the clearance average determined at 30 mg/100 ml plasma. In subject J C only two plasma levels were studied, at each of which four clearances were determined, and the average clearance of inulin at the higher level of plasma inulin was 7 per cent more than the average clearance at the lower level.

### DISCUSSION

The results obtained in the four experiments on those subjects in whom the renal clearance of inulin was measured at various levels of plasma inulin concentration, maintained approximately constant by continuous infusion of inulin, are in general agreement with the results obtained using the single injection method of administration. Between the limits of plasma inulin concentrations studied and on the basis of the claims of previous workers [Shannon and Smith 1935], straight lines representing the first approximation to the definition of the relationship between the variables and  $P$  were constructed. The method consisted

simply of averaging the determinations of  $uv$  and  $P$  at the highest and lowest limits at which the determinations were made, and obtaining the slope of the lines between these points by direct calculation. The points of intersection of the extrapolation of the lines on the ordinate ( $uv$ ) are then easily obtained. The results of this calculation are shown in Table VII. The points of intersection of the lines upon the ordinate

TABLE VII—THE POINTS OF INTERSECTION ON THE ORDINATES ( $uv$ ) AND THE SLOPES OF THE LINES RELATING THE RATE OF INULIN EXCRETION IN THE URINE ( $uv$ ) TO THE CORRESPONDING PLASMA INULIN CONCENTRATION ( $P$ ) FOR THE SUBJECTS GIVEN IN TABLES V AND VI. THE METHOD OF CALCULATION IS GIVEN IN THE TEXT

Subject	Slope	Point of intersection on negative ordinate
C.M.	1.218	- 14.28
	1.167	- 4.855
W.R.	1.676	- 8.30
J.C.	0.975	- 2.91

vary from -2.9 to -14.3. These amounts are of the same order of magnitude as the values calculated by the least squares method from data obtained following a single injection of inulin. The points of intersection are all negative, and the phenomenon of the smaller renal clearance of inulin with smaller plasma concentrations is apparently again due to the fact that the line truly representing the relationship between the two variables is either a curvilinear one passing through the origin, or (within the range studied) is linear but does not pass through the origin. The slope of a straight line from any point ( $P, uv$ ) experimentally determined, to the intersection of the co-ordinates (i.e.  $uv/P$ ), will again, for reasons already discussed, depend upon the level of  $P$  selected, and will show a tendency to fall as the plasma concentration of inulin approaches nothing.

The findings of Miller, Alving and Rubin [1940], in so far as they relate to the continuous infusion technique, are consistent with our own results, though the emphasis we place upon them is of a very different order. Omitting from consideration subject M.F. of Miller, Alving and Rubin [1940], because only one clearance period was studied at the high level of plasma inulin concentration, the percentage difference between the average values obtained for renal clearance at the high plasma level and the average values obtained at the low plasma level of inulin was calculated, and the results are shown in Table VIII. Apart from subjects B.H. and A.T., the average clearances are lower at the low plasma inulin levels by amounts comparable to our own results. Subject A.T., in whom the average clearance at the low plasma level is 35 per cent higher than the average clearance at the higher plasma level, has a very severely depressed function. Furthermore, the result is dubious, since the values for clearance at the same plasma level of inulin show a

TABLE VIII — DATA OF MILLER, ALVING AND RUBIN [1940] RESULTS OF INULIN CLEARANCE OBTAINED USING CONTINUOUS INFUSION OF INULIN AT DIFFERENT LEVELS OF PLASMA INULIN CONCENTRATION

Case	Plasma inulin concentration, mgm per 100 ml	Number of clearance periods	Average inulin clearance, ml per min	Percentage difference
D.K	45 - 46	2	110.5	- 13
	47.6 - 56.1	3	125.3	
M.B	9.9 - 10.9	2	114.0	- 11
	94.5 - 96.4	2	128.0	
B.E	3.3 - 3.6	2	121.0	- 15
	45.7 - 50.9	2	142.0	
B.H	5.7 - 5.7	2	115.0	+ 4
	12.4 - 13.9	2	105.0	- 5
	32.7 - 36.9	2	111.0	
L.M	4.2 - 4.5	3	86.1	- 22
	38.0 - 49.7	3	110.2	
V.S	4.7 - 4.8	2	86.5	- 17
	64.8 - 70.9	2	103.6	
E.S	4.1 - 4.4	2	57.5	- 14
	10.0 - 11.4	2	61.5	- 8
	70.2 - 71.8	2	66.5	
A.T	6.9 - 8.0	3	17.6	+ 35
	82.5 - 85.2	3	13.0	
M.C	7.4 - 7.7	3	20.6	- 6
	61.2 - 67.1	3	22.0	

variation of 31 and 36 per cent at the two levels—a variation which is larger than in any of the other subjects and which renders the comparison of the average clearances at the two levels questionable

Miller, Alving and Rubin [1940] interpreted their results as indicating no tubular reabsorption of inulin in the majority of these subjects, although they admitted the possibility of very slight reabsorption (about 1 mg/min) in some of their cases. This amount is estimated by assuming that the highest value for clearance represents the rate of glomerular filtration, and that the lower clearance at lower levels of plasma inulin concentration is due to reabsorption by the tubules. Although they attempt to justify this procedure on the grounds that if tubular reabsorption of inulin occurs it will obey the general rule of tubular behaviour and reach a maximum amount which is independent of the plasma level of inulin, the amount they calculate would in fact represent the amount reabsorbed by the tubules at inulin levels of the order of 5 mg/100 ml, provided there was indeed no reabsorption at the

higher levels. If tubular reabsorption of inulin does occur, and if it obeys the laws of tubular function which have been claimed for many other substances, the amount reabsorbed at the high levels of plasma inulin will be maximal, and may be much greater than the amount reabsorbed at levels of the order of 5 mg/100 ml plasma.

It is clear that, within the framework of the filtration-reabsorption theory of renal function, the slope of the line relating the rate of excretion of inulin and the plasma inulin concentration is the best estimate of a constant proportionality between the two variables at present available. If this slope is interpreted as representing the rate of glomerular filtration, the point of intersection of the line with the ordinate ( $uv$ ) represents the average influence of the tubules upon the filtered inulin between the levels of plasma inulin over which the line was determined.

The fact that the intersection occurs at a value for  $uv$  that is negative indicates that, on this theory, the tubules reabsorb inulin at a rate which, for the three subjects studied by the infusion method, ranges from 2.9 to 14.3 mg inulin/minute, amounts which are of the same order as those calculated for subjects studied by the single injection method.

If this procedure is adopted with the data obtained by Miller, Alving and Rubin [1940], using the infusion method, the straight lines calculated from the two extreme plasma levels of inulin are found to intersect the ordinate ( $uv$ ) at from 0.2 to -2 mg. This represents a degree of reabsorption which is less than is shown in our experiments either with the single injection method or the infusion technique. This disparity is probably due to the use, by these authors, of very low values of  $P$  (c. 5 mg inulin per 100 ml plasma) which, whatever the true form of the line  $uv=f(P)$ , would affect the slope of a straight line designed to fit the experimentally determined points.

## GENERAL DISCUSSION

The dependence of the renal clearance of inulin upon the plasma inulin concentration has been demonstrated by the results of experiments by both the infusion and single injection methods. The general correspondence between the results obtained by these two methods indicates that the relationship between the rate of renal excretion of inulin and the plasma concentration is not one of direct proportionality, but that the line representing the relationship of the two variables is either a straight line which does not pass through the origin, or a curvilinear one which passes through the point of origin and which possesses a form such that the first differential coefficient of  $uv$  with respect to  $P$  increases with an increase in  $P$ . The mode of construction of the line  $uv=aP-b$  and the absence of values for  $uv$  at very low plasma inulin levels prevent a definite choice between these possibilities. Under these circumstances

the systematic fall observed in the value for the renal clearance of inulin with falling plasma levels is explained

The published literature contains remarkably little concerning this phenomenon. Among the large number of workers who have employed the single injection technique, apparently Josephson and Lundahl [1943] alone report the phenomenon, stating that, following a single injection of inulin, the first two clearances measured after the injection are "statistically higher" than the values found during the third period. The results of Miller, Alving and Rubin [1940] by the infusion technique are compatible with our own, although, after considering the possibility of inulin reabsorption, they concluded that the amount must be negligibly small, their results by the single injection method did not show a regular fall of inulin clearance with falling plasma inulin, and no satisfactory explanation of their disagreement with our results can be offered.

The analysis summarised in Tables II, III and VII establishes the absence of a strict proportionality between the variables  $uv$  and  $P$ . The results may be considered with reference to two fundamental possibilities: either (1) inulin is completely filtered by the renal glomeruli and is neither reabsorbed nor excreted by the tubules, so that the inulin clearance is a measure of the rate of glomerular filtration, or (2) the renal clearance of inulin is not a measure of the rate of glomerular filtration.

On the assumption that inulin is completely filtered by the renal glomeruli and is subsequently unaffected by tubular activity, the relationship between  $uv$  and  $P$  should be given by the equation  $uv = AP$ , so that any other relationship (closely approximated by  $uv = aP - b$  over the range studied) must necessarily represent a distortion of the true relationship between the variables. Experiments have shown that the slight difference which exists between the concentration of inulin in arterial and venous plasma is not the source of this hypothetical distortion. It is possible, on the other hand, that inulin in the body or urine undergoes a change, either to a compound which would not be estimated by the method of analysis, or to one (e.g. fructose) which would contribute its complement of colour to the total colour reaction. Under the latter circumstances there is no reason to believe that the rate of excretion of this derived compound would bear the same relation to its blood concentration as that obtaining for inulin. It must, however, be admitted that there is no extant evidence which lends support to either possibility. On the contrary, the weight of evidence [Josephson and Lundahl 1943, Smith, 1937] indicates that inulin is not destroyed in the body and is completely excreted in the urine. Further, correspondence between the results obtained by using the single injection and the continuous infusion methods excludes the possibility that the relationship found is the



result of the dynamics of distribution of inulin after injection peculiar to one mode of administration. It has been shown that, following a single injection of inulin, equilibration between the plasma and the extravascular volume of distribution of inulin does not occur [Robson *et al.*, 1949]. That equilibration throughout the entire volume of distribution is attained during the normal period of continuous infusion is now doubtful in view of the reports of Guadino and Levitt [1949]. However, the uniform insignificance of the difference between the arterial and venous inulin concentrations during the experimental period shows that the value of  $\frac{uv}{P}$  is not affected by the non-equilibrium between plasma and extravascular fluid, even although there is a varying rate of inulin exchange between these two fluids—provided the exchange indicated by the arterio-venous difference in the general circulation truly represents the exchange in the kidney itself, and provided that the plasma is the sole source of the renal inulin. These provisos cannot be tested experimentally by methods at present available, but they are *a priori* sufficiently probable to justify the conclusion that a more likely interpretation of the inconstancy of inulin clearance must be sought.

The results are more satisfactorily interpreted on the basis of the second assumption that the renal clearance of inulin does not measure the rate of glomerular filtration. Accepting the filtration reabsorption theory of renal function in general, the relationship we have found between the plasma concentration of inulin and the rate of its renal excretion indicates the participation of the tubules in the removal of the substance from the body. If the true relationship is linear and represented by  $uv = aP - b$ , this is clear, and the intercept on the horizontal axis perhaps represents the value of  $P$  below which the whole of the filtered inulin is reabsorbed by the tubules. If, however, it is curvilinear, the position is analogous to that of glucose and ascorbic acid, and since the similar curves given by these substances are generally interpreted as resultants of glomerular filtration and tubular reabsorption, that given by inulin may well be interpreted in the same way, the amount of reabsorption being, however, relatively small.

If this be accepted, the slopes of the regression lines relating  $uv$  to  $P$  may then be considered to be the nearest approximation to the rate of glomerular filtration, since they represent the nearest approximation to the constant of proportionality between the two variables over the range studied. The assumption implied in this view is that the amount of inulin reabsorbed is constant and is independent of the plasma concentration of inulin above a certain critical value, and that the regression line is constructed for inulin concentrations greater than this.

It should be noted that below the "critical" value of  $P$  the amount of inulin present in the glomerular filtrate may be completely reabsorbed,

in this case the linear relation between  $uv$  and  $P$ , shown in figs 1 and 2, is the true one. If, however, the relation between  $uv$  and  $P$  is curvilinear, the most likely interpretation is that at low levels of  $P$  reabsorption is submaximal and functionally related to  $P$ , becoming maximal and constant when  $P$  exceeds some undetermined level.

In the experiments described in this paper, the data afford no means of determining the hypothetical critical level for the plasma inulin

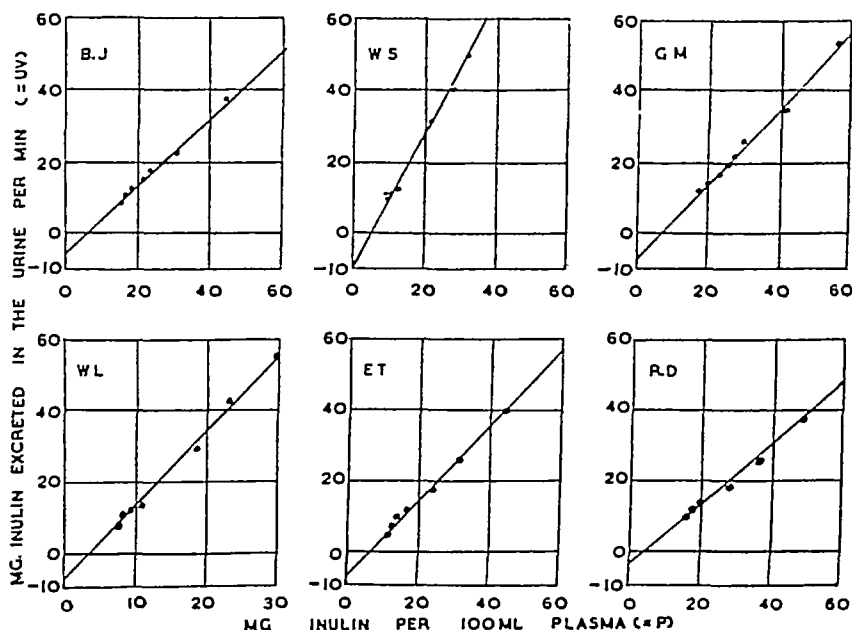


FIG 6—Values for the rate of inulin excretion ( $uv$ ) plotted against values for plasma inulin concentration ( $P$ ) in six of the subjects. The regression lines given in Table II are also drawn for the subjects.

concentration, that it is probably below 15 to 20 mg per 100 ml in most cases is strongly suggested by the diagrams given by plotting the individual  $uv$  values against the corresponding values for  $P$  and drawing the calculated regression line (fig 6), or from the data supplied in Tables II, III, V and VI.

In any case, the amount of reabsorption indicated by the intercept on the  $uv$  axis is minimal, since the use of values of  $P$  below the critical level would tend to reduce it.

Interpreting the line relating  $uv$  to  $P$  as representing reabsorption of inulin does not, however, necessarily involve accepting the concept of a fixed reabsorptive capacity for inulin at any plasma inulin level used in the experiments. If, however, it be supposed that at all levels there is a functional relationship between amount reabsorbed and plasma

concentration,<sup>1</sup> it becomes impossible to make any estimate, however crude, of either the glomerular filtration rate or the amount reabsorbed.

Evidently it is possible to interpret the results by supposing that some inulin is reabsorbed by the tubules. The considerations leading to this interpretation are based on the assumption that all the inulin appearing in the urine is derived solely from the plasma. It is, however, possible that the tubular cells gain inulin directly from the extravascular fluid and excrete it into the tubular lumen, if this were happening, the results of inulin clearance measurements could be obtained either as a resultant of this process and reabsorption (superimposed on glomerular filtration), or by glomerular filtration supplemented by this form of excretion alone.

In the latter circumstances the phenomenon of falling inulin clearance with falling plasma level of inulin would occur only if the increment added to the urine from the extravascular fluids was not proportional to the plasma level, but was diminishing at a rate progressively greater than the rate of disappearance of inulin from the plasma. For it is only under such circumstances that the urinary inulin per unit plasma concentration would fall. There is no direct information in regard to the renal extravascular fluid concentration of inulin following administration. However, the continuous increase in the volume of distribution of inulin (defined as  $\frac{\text{total inulin in body}}{\text{plasma concentration}}$ ) following a single injection [Robson *et al*, 1949] indicates that the average concentration of inulin in the extravascular volume of dispersion falls more slowly than the plasma concentration. Hence, on the basis of this indirect evidence, it is unlikely that such a rapidly diminishing increment of inulin from the extravascular compartment to the lumen does occur or is the explanation of the phenomenon.

It is obvious that the position with respect to the co-ordinates of the line relating  $uv$  to  $P$  can be considered in another way. We have considered the negative intercept of the  $uv$  axis, and have concluded that the existence is most easily interpreted by supposing it to be due to tubular reabsorption of inulin. This interpretation is based not only on the acceptance of the orthodox theory of renal excretory mechanism, but also on the assumption that the amount of inulin excreted in the urine during an infinitesimal period of time is derived from the plasma  $2\frac{1}{2}$  or 6 minutes previously. This may be wrong, the intercept may really be an artefact due to an error in the mode of relating  $uv$  to  $P$  in time. If one accepts the view that  $\frac{uv}{P}$  is constant for all

<sup>1</sup> This, in view of the behaviour of other substances, is unlikely, and the least unlikely relationship, a direct proportionality between amount reabsorbed and plasma inulin concentration, would give a straight line passing through the origin.

values of  $P$ , then the positive intercept on the  $P$  axis (OC in fig 3) shows the existence of an error, and it is necessary to suppose that the "lag" between  $P$  and  $uv$  is not  $2\frac{1}{2}$  or 6 minutes but much more. The available evidence derived from the dependence of  $uv$  upon  $P$  in infusion experiments, and upon measurements of the appearance in the urine of dyes injected intravenously, so strongly suggests the validity of the shorter time used by ourselves and other observers, that we adhere to the view that our results indicate tubular reabsorption of inulin, admitting, however, that this adherence is based on our estimate of probabilities rather than on conclusive proof.

The whole of this discussion has been based on the view that the orthodox filtration-reabsorption theory of renal function is fundamentally correct. This, conceivably, may not be the case, but the evidence in favour of the theory is so strong [Smith, 1937] that this remote possibility need not seriously be considered. What does seem likely is that substances which are completely uninfluenced by tubular activity may be more ideal than real.

The results which we have described for inulin between plasma levels of about 15 mg/100 ml and 100 mg/100 ml, using the single injection and infusion methods, show considerable resemblance to the results obtained by many workers with exogenous creatinine [Winkler and Parra, 1937, Findley, 1938, Cope, 1931, Dominguez and Pomerene, 1934]. This extends to the phenomenon, not included in the results presented earlier in this paper, of a rise in the clearance of inulin following a second injection of the substance. This has been found true for creatinine by Shannon and Ranges [1941], and we have regularly observed it in the case of inulin. The marked depression of the clearance of creatinine following extreme elevation of the plasma concentration, which is interpreted as an indication of tubular excretion, does not appear to find a parallel in so far as inulin is concerned. The results of Shannon and Smith [1935] were likely to obscure any slight elevation or depression of clearance, but any marked tendency one way or the other would have been observed.

Experiments with still higher inulin levels than these workers used would be difficult because of the limitations imposed by the solubility of inulin. Nevertheless, the falling values of the renal clearance of inulin with falling plasma concentrations, the rise in the values which follow a second injection of inulin after the clearances have fallen, and the maintenance of a constant clearance when the plasma level remains constant, all suggest that the explanation offered by Shannon and Ranges [1941] for these phenomena in relation to creatinine is less likely than that afforded by tubular activity.

In view of the findings reported in this paper, it becomes important to decide the implications of the results in relation to the interpretation of values obtained for inulin clearance derived from experiments con-

ducted either with inulin concentrations of the order of 25 mg inulin/100 ml plasma, as advocated by Goldring and Chasis [1944] when the infusion method is used, or the plasma inulin concentrations normally below 60 mg/100 ml which are attained when the single injection technique is used. In the absence of any independent estimate of glomerular filtration in these subjects, precise definition of the implications is

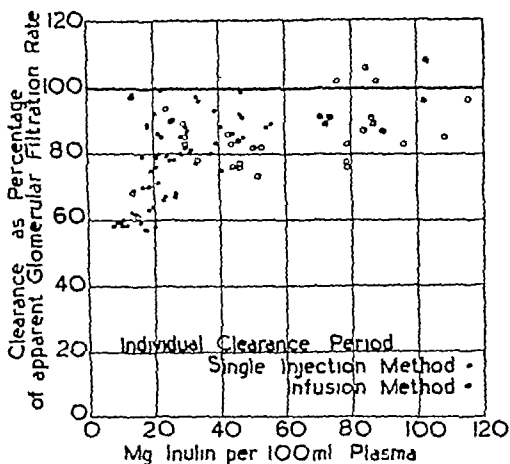


FIG. 7—Individual values of the clearance of inulin for the fifteen subjects expressed as a percentage of the slope of the regression lines given in Table II (data from single injection experiments) and Table VII (data from infusion experiments) 100 = the slopes of the regression lines and is taken as the apparent glomerular filtration rate

impossible. The values obtained at various levels of plasma inulin concentration, using the expression  $\frac{uv}{P}$ , may, however, be compared with slopes of the lines relating  $uv$  to  $P$  calculated from the experimental data. These slopes may be taken to be the nearest approximation to the estimate of glomerular filtration available in these subjects.

In this way the degrees of under-estimation of these slopes obtained by use of single values of  $uv$  and  $P$  in an individual estimation of clearance may be measured. In fig. 7, values for inulin clearance for each individual period, expressed as a percentage of the value of filtration as estimated by the slope of the regression lines, are plotted against the venous plasma inulin concentration for the corresponding period. The solid dots represent the individual values for clearance obtained in subjects using the single injection method and given in Table I, the open circles represent individual values for clearance of the subjects in whom determinations were made using the infusion method and given in Tables V and VI. In all subjects the values for clearance represented by the slopes of the lines relating  $uv$  to  $P$  are taken as 100 per cent.

As may be anticipated from the results already presented, the

values of  $P$ , then the positive intercept on the  $P$  axis (OC in fig 3) shows the existence of an error, and it is necessary to suppose that the "lag" between  $P$  and  $uv$  is not  $2\frac{1}{2}$  or 6 minutes but much more. The available evidence derived from the dependence of  $uv$  upon  $P$  in infusion experiments, and upon measurements of the appearance in the urine of dyes injected intravenously, so strongly suggests the validity of the shorter time used by ourselves and other observers, that we adhere to the view that our results indicate tubular reabsorption of inulin, admitting, however, that this adherence is based on our estimate of probabilities rather than on conclusive proof.

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4 The lack of proportionality is shown not to be due to the sampling of venous blood as an estimate of arterial plasma inulin concentrations

5 The dependence of the magnitude of the renal clearance of inulin upon the plasma concentration of inulin is found also in three subjects using the continuous infusion method of administration. This excludes the possibility that the results obtained, following a single injection of inulin, are distortions of a simple proportional relationship consequent upon the dynamics of distribution of inulin in the body following a single injection

6 The results of the experiments, and those of other workers, are held to suggest strongly that, in man, inulin is reabsorbed by the tubules to a significant extent. Hence the use of inulin clearance as a measure of glomerular filtration rate involves a systematic error, which is estimated to be about 15 per cent under the usual circumstances

#### ACKNOWLEDGMENTS

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expression  $\text{Clearance} = \frac{uv}{P}$  provides values for clearance which are in general smaller than the values represented by the slopes of the lines. The scatter diagram shows, further, that the degree of under estimation of the slopes increases as values of  $P$  diminish. This is again to be expected from the fact that the lines relating  $uv$  to  $P$  intersect the negative ordinate. Considering only values for clearance obtained by using plasma inulin concentrations of 20 mg per cent or more, the average under-estimate of the slopes of the lines of the 66 clearance values obtained with the single injection method is 17 per cent, and the average under-estimate of the slopes of the lines relating  $uv$  to  $P$  using the infusion method is 13 per cent. Considered together (100 clearance periods), the expression  $\frac{uv}{P}$  provides values which are, on the average,

85 per cent of the values obtained by calculating the slopes of the lines.

It is seen from these figures and from the scatter diagram that, above plasma inulin concentrations of 20 mg per cent, the under-estimate is of a similar order irrespective of the method of administration. In emphasising this point, however, it is to be remembered that the slopes of the lines relating  $uv$  to  $P$  are themselves influenced by the level of  $P$  employed in the calculation, and that the values of  $P$  attained by the infusion method are higher on the whole than those attained by a single injection of inulin. Fig 5 illustrates how the slope of this line is altered by incorporating lower values of plasma inulin concentration.

It is clear that the under-estimate will be of the same order if the formula devised by Robson *et al* [1949] is employed, since it has already been shown that although this formula was developed on the basis of constancy of the inulin clearance, it provides a value which is an average of what is an apparently changing clearance between the two plasma levels over which it is applied.

## SUMMARY

1 Following a single intravenous injection of inulin, values for the renal clearance of inulin ( $C_{in} = uv/P$ ) show a steady decline, with diminishing values of plasma inulin concentrations.

2 Employing values of  $P$  6 minutes before the mid-point of the clearance period, in place of values  $2\frac{1}{2}$  minutes before the mid point, does not eliminate this phenomenon.

3 Employing the formula  $C_{in} = uv/P$ , the fall in values for clearance is due to the fact that the renal excretion of inulin ( $uv$ ) is not proportional to the plasma level ( $P$ ), since the imposition of a linear relationship between the two variables results in lines which deviate significantly from the point of origin.



# EXCRETION OF HISTAMINE IN HUMAN URINE By H M ADAM From the Departments of Pharmacology and Therapeutics, University of Edinburgh

(Received for publication 4th November 1949)

It is now known that histamine is a normal constituent of mammalian urine [Anrep, Ayadi, Barsoum, Smith and Talaat, 1944, Gaddum, 1948, Rosenthal and Tabor, 1948, Hunter and Dunlop, 1948, Urbach, 1949] In human urine it occurs mainly in a conjugated and inactive form free histamine is found only in traces Recently, Urbach [1949] has obtained evidence by paper partition chromatography that the conjugate is probably identical with N-acetyl histamine [4( $\beta$ -acetyl-amino ethyl)imidazole]

In the dog, 3 to 5 per cent of histamine given by mouth is excreted as the conjugate When, however, histamine is injected subcutaneously it appears in the urine in the free form [Anrep *et al*, 1944]

The object of the present investigation was to study in man the excretion of histamine given orally and by intravenous infusion At the same time an attempt was made to discover whether, under the conditions of infusion, an increase of the histamine concentration could be detected in the blood as well as in the urine

Rose [1940] failed to detect an increase in samples of whole blood after the subcutaneous injection of 1 mg histamine base in man Emmeln, Kahlson and Wicksell [1941] observed in the dog that a measurable rise of the plasma histamine occurred only when the rate of intravenous infusion was 2-5  $\mu\text{g/kg/min}$  According to Weiss, Robb and Ellis [1932], this rate of infusion would be likely to have toxic effects in man

The present study was carried out on four men, designated D H, McG, McF and M W

## METHODS

### 1 Pharmacological

#### (a) Extraction of Histamine from Urine

A simplified form of the charcoal method [Anrep *et al*, 1944] was used Two stages in the extraction procedure were omitted the final alcoholic extraction, and the treatment of the aqueous solution of the



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NaOH delivered from a fine capillary. Make the volume of the suspension up to 5 ml with 5.85 per cent NaCl and centrifuge at 2000 r p m for 5 minutes. Decant the supernatant and, if need be, make a final adjustment of the pH before proceeding to the assay.

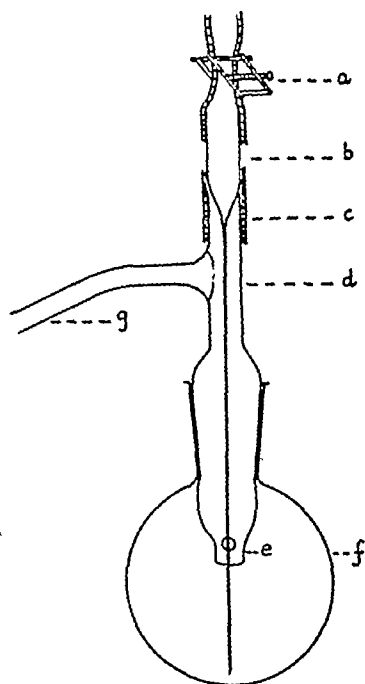


FIG 1—Apparatus for evaporation of extracts (a) Screw clip on rubber connection, (b) glass capillary (c) rubber connection, (d) ground glass cone with bubble trap (e) and side arm (g). This part (d, e, g) is adapted from a distillation tube (M15, Quickfit and Quartz). (f) 150 ml spherical flask (B24, Quickfit and Quartz). The side arm (g) is inserted into a manifold which is connected to a trap and water pump.

This method was tested by recovering histamine acid phosphate added to different samples of urine. A control sample of each urine was extracted at the same time. In the present experiments the recovery of conjugated histamine was assumed to be the same as for histamine added to urine.

#### (b) *Extraction of Histamine from Blood*

Code's modification [1937] of the method of Barsoum and Gaddum [1935] was used. Citrated or heparinized blood samples of 10 ml were centrifuged at 3000 r p m for 15 minutes and the plasma and cells extracted separately. The final volume of the solution of each extract used for the assay was 10 ml.

extract with alumina. Further, the elution was carried out at a raised temperature, which obviated the need for repeated washing of the charcoal with each lot of acid alcohol. No relaxing substance was encountered in extracts of human urine prepared by this method.

*Reagents*—These were similar to those employed in the original method. The activated charcoal was obtained from Messrs Sutchiffe, Speakman & Co Ltd, Leigh, Lancs. It contained iron which was removed by boiling in concentrated HCl (A.R.) for 15 minutes in the proportion of 1 g charcoal to 10 ml of acid. The charcoal was then washed with distilled water until free from acid, and dried in a desiccator.

Absolute ethyl alcohol was purified by refluxing with solid NaOH and redistillation.

*Procedure*—Take 10 ml of filtered, neutral urine and shake it with 0.25 g charcoal in a centrifuge tube for five minutes. Centrifuge the suspension at 2000 r.p.m. and discard the supernatant. Resuspend the charcoal in 10 ml distilled water and repeat the centrifuging. After decanting the supernatant, add 10 ml of 95 per cent acid alcohol (95 ml absolute alcohol + 3 ml 10N HCl + 2 ml distilled water) to the charcoal and stir with a glass rod to obtain thorough mixing. Immerse the tube for 15 minutes in a water-bath at 60°–65° C and stir the suspension from time to time. Next decant the suspension on to a No. 12 Whatman filter-paper and filter by gravity into a 50 ml measuring cylinder. Wash the tube clean with three successive lots, each of 10 ml of warm 75 per cent acid alcohol (75 ml absolute alcohol + 3 ml 10N HCl + 22 ml distilled water), and pour each lot on to the layer of charcoal on the filter-paper. One lot can be conveniently warmed up, while the other is filtering, by placing the tube in the water-bath for 3 minutes. Heat is applied to the filter funnels to speed up filtration. The eluate has a final volume of about 35 ml, and can be stored overnight at 0° C without loss of activity. Divide the eluate into two equal portions and take each to dryness. The evaporations are carried out under reduced pressure on a water-bath at 60° C. Fig. 1 shows the type of apparatus used. Wash one residue with 10 ml absolute alcohol and set it aside for the estimation of free histamine. The other residue is hydrolysed in the presence of 10 ml 10N HCl (A.R.). For this purpose, fit an air condenser to the flask and immerse the flask in a boiling water-bath for 1½ hours. Then distil off the acid and wash the hydrolysed extract with 10 ml absolute alcohol. At this stage the dried extracts can, if necessary, be stored in a desiccator for several days without loss of activity. Finally, take up the two extracts, hydrolysed and unhydrolysed, in three lots of 1.5 ml each of hot distilled water. Scrape the flask clean with a glass rod fitted with a rubber policeman. Transfer each wash by dropping pipette to a 10 ml graduated centrifuge tube. Add one drop of a 0.1 per cent solution of neutral red to the suspension and neutralise it with 1–2 drops of 2N

immediately stored at 0° C and, if necessary, acidified Under these conditions free histamine is stable for at least 24 hours, and conjugated histamine for several weeks In an earlier experiment (D H) 24-hour collections were made, and chloroform (approx 0.5 per cent) was added as a preservative

A preliminary routine examination of the urine, and an experiment to test for the recovery of added histamine, were carried out in each subject In none was there any evidence of abnormality of the urine or of failure to recover the expected amount of histamine

## RESULTS

### 1 *Recovery of Histamine added to Urine*

Table I contains a summary of the results of experiments on different urines to which histamine was added in concentrations of 100 and 500  $\mu\text{g}$  per litre Table II shows one set (500  $\mu\text{g}$  per litre) of results in detail The initial value is that of the control sample of urine, the final, of the urine after the addition of histamine, the difference represents the recovery, and is expressed in the last column as a percentage of the amount added Within each series, the difference between the means of the recoveries for free and total histamine is not statistically significant Hence the extracts did not differ in their content of substances that might be expected to interfere with the assay of histamine The recovery of histamine added to the test solutions after these had been assayed confirmed this view the mean recoveries were 91 per cent (range 83–103) and 92 per cent (range 85–95) for the unhydrolysed and hydrolysed tests respectively These values fell short of 100 per cent recovery, but were mostly within the limits of the experimental error of the assay If inhibitory substances were present in the extracts, they did not wholly account for the incomplete recovery of histamine added to urine Experiments on the extraction of histamine in similar concentrations from Tyrode solution and distilled water showed that a loss of 10 to 15 per cent might be expected, because of incomplete elution from the charcoal There is no evidence that conjugated histamine, in the concentrations encountered in the present experiments, interferes with the recovery of free histamine

TABLE I—RECOVERY OF HISTAMINE ADDED TO URINE

Concentration micrograms per litre	100		500	
Number of estimations	13		12	
Percentage recovery	Total	Free	Total	Free
Mean	69.9	74.8	86.2	83.6
S D for single observation	15.4	14.2	9.1	7.7
S E of mean	4.3	3.9	2.6	2.6

### (c) *Estimation and Identification of Histamine*

The histamine values are all calculated in terms of the base, on the assumption that this represents one-third of the weight of the phosphate

The extracts were tested on a strip of guinea-pig ileum suspended in 2 ml Tyrode's solution containing atropine ( $0.1 \mu\text{g/ml}$ ) and were compared with a standard solution of histamine acid phosphate (British Drug Houses Ltd). Mepyramine maleate (May & Baker Ltd) was used as a test for histamine in the extracts [Reuse, 1948]

## 2 *Clinical*

### (a) *Administration of Histamine*

*Oral*—The dose of histamine was dissolved in 0.9 per cent NaCl to give a concentration of  $0.33 \text{ mg/ml}$ . The solution was run slowly into the stomach through a Ryle tube. The subject was in a fasting state and had previously emptied the bladder.

*Intravenous Infusion*—After being at rest for 20 minutes the subject voided the bladder, and was immediately connected by one arm to a saline intravenous drip infusion. A control sample of venous blood was removed from the other arm. The saline infusion was then stopped, and a solution containing  $10 \mu\text{g}$  of histamine per ml of 0.9 per cent NaCl was infused by gravity from a graduated aspirator bottle of 1 litre capacity. To this was connected a drip chamber previously calibrated by relating drop rate to volume flow in ml per minute. The flow rate was regulated by adjusting a screw-clip placed between the aspirator bottle and the drip chamber. In this way, quantities of 3.3 and 5 mg of histamine were infused in 2 to 3 hours. The total amount infused after an interval of time could be read approximately on the scale attached to the bottle, and the rate of infusion (in  $\mu\text{g/min}$ ) estimated at any moment by counting the drop rate per minute.

### (b) *Effects of Histamine*

The pulse rate and arterial blood-pressure were recorded at intervals of 5 to 10 minutes before and during the intravenous infusion of histamine. The onset and severity of headache, and extent of flushing, were also noted. The infusion rate was increased at varying times, but usually not beyond the limit which produced intolerable headache or a large fall in blood-pressure. The second venous blood sample was removed towards the end of the infusion.

### (c) *Collection of Urine Samples*

The urine was collected directly into chemically clean, stoppered 750 ml conical flasks at intervals of 6 hours. The sample was



the limits of error ( $P=0.95$ ) for a single observation are 47–102  $\mu\text{g}$  per litre. When the concentration is 500  $\mu\text{g}$  per litre the mean recovery is 83.6 per cent or 418  $\mu\text{g}$  per litre, and the corresponding limits of error are 342–495  $\mu\text{g}$  per litre.

The amount of free histamine normally present in the urine is less than can be estimated within the limits of error already mentioned. After the administration of large doses of histamine, however, the concentration of free or conjugated histamine in the urine may be expected to reach 100  $\mu\text{g}$  per litre, and even to exceed it.

### *Excretion of Histamine in Urine*

#### *(a) After Oral Administration*

This was studied in subjects D.H., McG. and M.W., who were on an ordinary hospital diet. D.H. received two doses of histamine and the

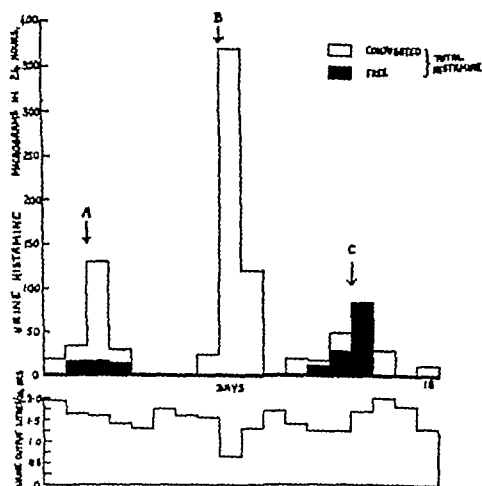


FIG. 2.—Subject D.H. Total histamine excretion following (A) 67 mg histamine orally, (B) 133 mg histamine orally, (C) 3.3 mg histamine by slow intravenous infusion.

urine was collected daily. McG. and M.W. were given single doses, and the urine was collected at six-hourly intervals to find out how quickly conjugated histamine was formed and excreted. The results of these experiments are given in Tables III, IV and V, and are shown graphically in Figs. 2 and 3.

The values obtained for McG. and M.W. show that about 50 per cent of the conjugated histamine which appears in the urine does so in the first 6 hours, that excretion proceeds exponentially and is nearly

TABLE II—RECOVERY OF HISTAMINE ADDED TO URINE

Concentration 500  $\mu\text{g}$  per litre

Micrograms histamine per litre urine

Expt	Urine	Histamine			
		Initial	Final	Difference	Percentage recovery
1	Total	50	530	480	96
	Free	50	500	450	90
	Total		530	480	96
	Free		500	450	90
} duplicate					
2	Total	10	450	440	88
	Free	< 10	450	450	90
3	Total	45	540	495	99
	Free	20	500	480	96
4	Total	30	400	370	74
	Free	10	400	390	78
5	Total	30	400	370	74
	Free	10	400	390	78
6	Total	15	500	485	97
	Free	< 10	400	400	80
7	Total	< 10	400	400	80
	Free	< 10	400	400	80
8	Total	15	460	445	89
	Free	< 10	400	400	80
9	Total	30	400	370	74
	Free	< 10	370	370	74
10	Total	15	425	410	82
	Free	< 10	466	466	93
11	Total	30	430	400	80
	Free	< 10	400	400	80

It should be mentioned that the unhydrolysed extract may exhibit actions which disappear after treatment with concentrated HCl. These are seen after a full dose (0.2 ml) of the extract is added to the bath: there is some frothing, a slight increase in the rhythmic activity of the gut, and an unusually large contraction after washing out.

## 2 Accuracy of the Method

The method can be used to estimate histamine in human urine in concentrations of not less than 100  $\mu\text{g}$  per litre. For example, the mean recovery for free histamine at this level is 74.8  $\mu\text{g}$  per litre, and

TABLE III—EXCRETION OF HISTAMINE IN URINE PER 24 HOURS

D.H. *æt* 40 B W 80 kg

Day	Urine vol ml	Histamine $\mu$ g		Percentage of dose excreted per 24 hours
		Free	Conjugated	
1	1950	< 20	> 20	
2	1650	16	34	
67 mg histamine in 200 ml 0.9 per cent NaCl orally				
3	1600	16	112	0.17
4	1400	14	14	
5	1300	< 13	0	
6	1750	< 18	0	
7	1600	< 16	0	
8	1550	< 15	> 9	
133 mg histamine in 400 ml 0.9 per cent NaCl orally				
9	650	7	363	0.27
10	1300	13	104	
11	1700	< 17	0	
12	1400	< 14	> 7	
13	1250	12	7	
14	1250	30	20	
3.3 mg histamine in 330 ml 0.9 per cent NaCl infused intravenously in 120 minutes				
15	1700	85	0	2.60
16	2000	< 10	> 30	
17	1750	< 17	0	
18	1300	< 13	> 13	

TABLE IV—EXCRETION OF HISTAMINE IN URINE PER 6 HOURS  
BEFORE AND AFTER ORAL DOSEMcG *æt* 48 B W 60 kg

Hours	Urine vol ml	Histamine $\mu$ g		Percentage of dose excreted
		Free	Conjugated	
24-18	380	< 4	> 2	
18-12	295	< 3	> 7	
12-6	400	< 4	> 3	
6-0	390	< 4	> 4	
133 mg histamine in 400 ml 0.9 per cent NaCl				
0-6	500	17	483	0.67
6-12	406	8	262	
12-18	430	4	74	
18-24	414	4	37	
24-30	335	< 3	> 9	
30-36	405	< 4	> 6	

complete in 24 hours. It may also be noted that the excretion of free histamine is slightly increased in the first 6 hours. In man, as in the dog, the amount of conjugated histamine excreted is independent of changes in the urinary output.

(b) *During and after Intravenous Infusion*

This was studied in subjects D H, McG and McF, and the results are shown in Tables III, VI and VII, and in figs 2 and 3. In subject

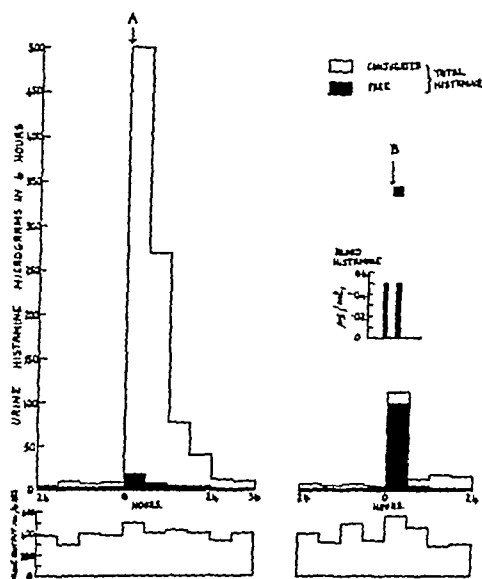


FIG. 3—Subject McG. Total histamine excretion following (A) 133 mg histamine orally, (B) 5 mg histamine by slow intravenous infusion.

McF, whose excretion was comparatively low, difficulties arose in the control of the infusion rate for a period it ran at  $0.82 \mu\text{g/kg/min}$  and the blood-pressure fell steeply. It may be that during this period of low blood-pressure urine formation was suppressed, and that histamine, which would have been excreted in the urine, disappeared in other ways. Anrep *et al* [1944] found this to be so in the dog after large doses were injected subcutaneously. D H and McG each excreted about the same proportion of the dose given. McG's excretion curve (fig 3) shows that all the histamine excreted was present in the first six-hourly sample. McF continued to excrete small quantities in the succeeding six-hourly samples, but these are of doubtful significance.

*The Blood Histamine during Infusion*

Venous blood samples were removed before and during the infusion from subjects McG and McF. No increase in the histamine content of the cells or of the plasma was detected. At the time when the infusion sample was removed the rates were approximately 0.48 and 0.28  $\mu\text{g}/\text{kg}/\text{min}$  for McG and McF respectively, and the drug was producing characteristic effects on the circulation.

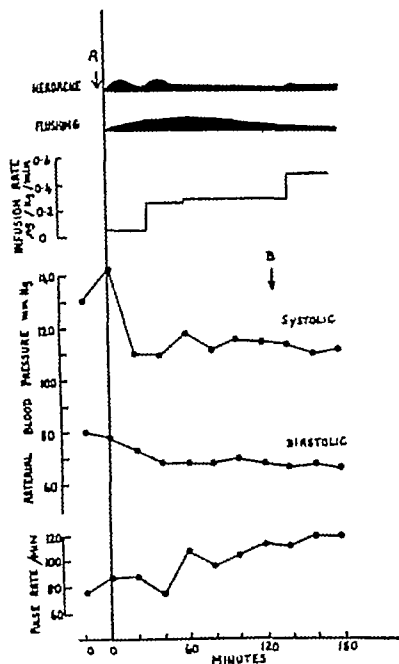


FIG 4—Subject McG. Effects of 5 mg histamine given by slow intravenous infusion. (A) Remove control blood sample (10 ml) and start histamine infusion. (B) Remove second blood sample (10 ml).

*Other Effects of the Infusion*

When histamine is infused intravenously for several hours, its action on the cardiovascular system frequently diminishes [Weiss *et al*, 1932]. In the present study it was confirmed that the intensity of the skin flush and of the headache decreased during the course of the infusions, which were given at an increasing rate (fig 4). Weiss *et al* ascribe this effect not only to the chemical inactivation of histamine but also to a physiological inactivation through the formation of a substance or substances antagonistic to histamine. In a few incidental observations it has not been possible to demonstrate that either the blood histaminase or the plasma adrenaline is increased at the end of an infusion.

TABLE V—EXCRETION OF HISTAMINE IN URINE PER 6 HOURS  
BEFORE AND AFTER ORAL DOSE

M W aet 57 B W 78 kg

Hours	Urine vol ml	Histamine $\mu$ g		Percentage of dose excreted
		Free	Conjugated	
24-18	770	< 8	> 15	
18-12	740	7	15	
12-6	405	4	9	
6-0	405	4	9	
133 mg histamine in 400 ml 0.9 per cent NaCl				
0-6	520	18	734	
6-12	330	8	404	
12-18	450	< 4	> 153	1.0
18-24	580	< 5	> 55	
24-30	695	7	25	
30-36	695	7	25	
36-42	420	< 4	> 12	
42-48	420	< 4	> 12	

TABLE VI—EXCRETION OF HISTAMINE IN URINE PER 6 HOURS  
BEFORE AND AFTER INTRAVENOUS INFUSION

McF aet 32 B W 68 kg

Hours	Urine vol ml	Histamine $\mu$ g		Percentage of dose excreted	Blood histamine $\mu$ g per ml	
		Free	Conjugated		Cells	Plasma
24-18	540	< 5	0			
18-12	470	< 4	> 5			
12-6	95	< 1	8			
6-0	1020	< 10	> 7		0.045	< 0.01
5 mg histamine in 500 ml 0.9 per cent NaCl infused intravenously in 180 minutes						
0-6	318	30	5	0.6	0.04	< 0.01
6-12	335	7	7			
12-18	250	5	0			
18-24	395	4	2			

TABLE VII—EXCRETION OF HISTAMINE IN URINE PER 6 HOURS  
BEFORE AND AFTER INTRAVENOUS INFUSION

McG aet 48 B W 68 kg

Hours	Urine vol ml	Histamine $\mu$ g		Percentage of dose excreted	Blood histamine $\mu$ g per ml	
		Free	Conjugated		Cells	Plasma
24-18	400	< 4	> 4			
18-12	310	3	3			
12-6	488	< 5	> 2			
6-0	325	< 3	> 2		0.05	< 0.01
5 mg histamine in 500 ml 0.9 per cent NaCl infused intravenously in 170 minutes						
0-6	560	100	12	2.2	0.05	< 0.01
6-12	454	5	7			
12-18	278	< 3	> 16			
18-24	300	3	12			

## SUMMARY

1 A simplification of the method of Anrep *et al* for the estimation of histamine in human urine is described

2 It is not possible by this method to obtain a reliable estimation of the amount of free histamine normally present in human urine, but the effects of the administration of histamine may be followed .

3 When 133 mg of histamine was given by mouth to three men, it was mainly conjugated histamine that appeared in the urine

4 When 3.5-5 mg was infused intravenously in three men, free histamine appeared in the urine

5 In two of these experiments no changes were detected in the histamine content of the plasma or cells obtained from the antecubital vein It is concluded that investigations of the excretion of histamine are more likely to give information about the release of histamine in the body than investigations on venous blood

## ACKNOWLEDGMENTS

I wish to thank Professor J H Gaddum for advice and criticism, Professor D M Dunlop for permission to make observations on patients in his wards, Dr R B Hunter for valuable assistance with the intravenous infusions, and Dr Kapeller-Adler for estimations of the blood histamine and histaminase

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## DISCUSSION

The results obtained in man for the excretion of administered histamine confirm in a general manner those recorded by Anrep *et al* [1944] for the dog. When histamine was given by mouth, the excretion of the conjugate varied between 0.17 and 1 per cent of the dose, in the dog, Anrep *et al* found 3 to 5 per cent. In man, as in the dog, there was only a slight increase of the free histamine which appeared in the first six-hourly sample. When histamine was given by intravenous infusion in doses of 3.3–5 mg, the excretion in man varied between 0.6 and 2.6 per cent, in the dog the same authors found 1.75 per cent (calculated from their figures) after subcutaneous injection of 10 mg of the acid phosphate, but they were unable to find a relation between the dose injected and the proportion excreted because of anuria of variable duration. It is confirmed also that free histamine is normally present, but only in traces, in human urine.

According to Urbach [1949], conjugated histamine is formed in the intestine by the action of bacteria. Some of it is absorbed and appears in the urine. In paper chromatograms of extracts of urine and faeces which were made after the ingestion of histamine diphosphate, it behaved like acetyl histamine [4( $\beta$ -acetylamino-ethyl)imidazole]. The chromatogram of urine collected at 1½ hours, and of a stool collected six hours, after an oral dose of histamine diphosphate showed a pronounced acetyl histamine spot. These findings agree with the results of the present experiments, which show that about half of the conjugated histamine excreted in 24 hours appears in the urine in the first six-hourly sample.

The conjugation of histamine has also been found to occur in the tissues of the rat [Millican, Rosenthal and Tabor, 1949]. These authors injected histamine subcutaneously in rats deprived of the alimentary canal below the level of the stomach, and obtained conjugated histamine in the urine. In man, however, there is no evidence to suggest that conjugation takes place outside the alimentary tract.

It appears that free histamine infused intravenously is more easily detected in the urine than in the venous blood, probably because it is concentrated in the kidneys. Failure to detect it in the venous blood by methods at present available confirms the findings of Rose [1940] and Emmeln *et al* [1941]. In seeking evidence for the theory that liberated or newly formed histamine contributes to the pathogenesis of certain diseases, it may be useful to examine the urine as well as the blood.



VASOMOTOR NERVE FIBRES TO THE MAMMARY GLANDS  
OF THE CAT AND DOG By J L LINZELL, Institute of  
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It has not been clearly established that there are vasomotor fibres to the mammary gland, for there has been no direct study of this subject. Earlier work which suggested the presence of such nerves to the mammae has been reviewed by Basch [1903]. More recently, Cannon and Bright [1931], Bacq [1932] and Clark [1933] found that a small proportion of animals showed lactational deficiencies as a belated result of partial or complete sympathectomy in the dog, cat and rat, and biochemical studies in the cow have shown that great and sudden changes in the permeability of the mammary blood-vessels, and temporary cessation of milk secretion, can occur in response to fear and pain [Graham, Kay and McIntosh, 1936, Shaw and Petersen, 1939]. St Clair [1942] noted that cutting the inguinal nerve or removal of the lumbar sympathetic chain caused an increase in the temperature of the udder in the cow, and, since this work was started, Peeters, Coussens and Sierens [1949] have briefly reported that electrical stimulation of the same nerve decreased the venous outflow of the isolated cow's udder perfused at constant pressure.

#### METHODS

*Animals*—Cats were used as the main experimental animal after trial experiments had shown that the mammary glands of the rabbit, rat and guinea-pig were too small for the apparatus available. The pregnant animals, selected by abdominal palpation from all the female cats received at the laboratory at all times of the year, were maintained on an adequate diet and used for experiments at various stages (2–73 days) after parturition. A smaller number of cats, for which the date of parturition was unknown and which were still lactating or had just weaned their kittens, were also used. A few dogs, whose mammary glands were commencing to involute after the normal lactation period, and one pseudo-pregnant lactating bitch, were also tested.

*Anæsthesia*—To avoid undue excitement, the animals were anæsthetised before being separated from their kittens. The anæsthetic, which



Extensive arterial and venous anastomoses between the branches of the main vessels, revealed by Neoprene casts (made by injection at physiological pressures), allow either pair to be clamped for cannulation without entirely depriving any gland of its blood supply. The subcutaneous abdominal vessels are the most easily approached surgically, and for this reason glands 3 and 4 have been chiefly studied.

(b) The mammary glands are innervated by the ventral cutaneous branches of the thoracic and lumbar spinal nerves, which mostly enter them from the lateral aspect. The inguinal gland 4 receives in addition branches from the external spermatic nerve, which enters it with the subcutaneous abdominal vessels. This nerve is the continuation of the genito-femoral (genito-crural), which arises from the fourth and fifth lumbar nerves. It passes through the inguinal canal in front of the external pudic vessels.

### *Perfusion of the Isolated Mammary Gland*

This was based on the method of Richards and Plant [1916], in which a pump was placed in series with the animal and the mammary gland,

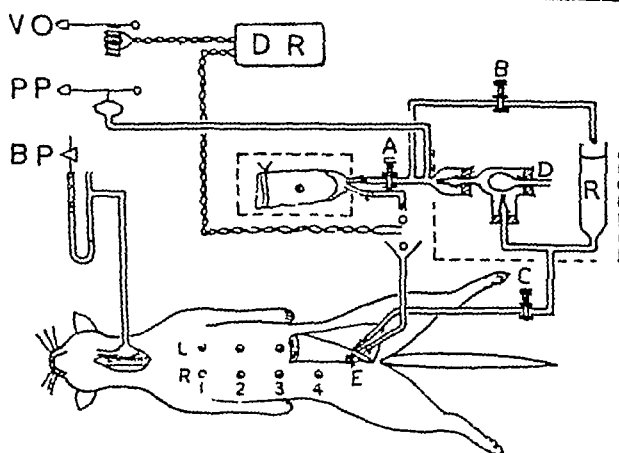


FIG 1a.—Diagram of the perfusion method, indicating perfusion of the inguinal mammary gland L 4

- BP = blood pressure recorder from carotid artery
  - VO = venous outflow recorder
  - PP = perfusion pressure recorder
  - DR = drop recorder
  - E = stump of the subcutaneous abdominal vessels
  - D = rubber pulsator connected to a Dale Schuster pump (not shown)
  - R = blood reservoir
  - C = screw clips controlling blood inflow from cat
  - A and B = screw clips controlling shunt (see text)
- The broken lines indicate the parts enclosed in warmed chambers

using the animal's own arterial blood as the perfusate (see fig 1a). It was adopted in an effort to overcome the objections to the methods dispensing with the animal [Petersen, Shaw and Visscher, 1941, Peeters and

was injected into the right cephalic vein, was one of the following (a) 6.45 per cent pentobarbitone in 10 per cent alcohol (Nembutal, Abbott) (b) 2 per cent chloralose in normal saline (c) Pentobarbitone induction followed by 20 per cent urethane in normal saline (d) A mixture of 3.3 per cent chlorose and 33 per cent urethane in normal saline. By this method the dosage required to induce deep anaesthesia varies with the condition of the animal and the rate of injection (the first two-thirds of the computed dose was injected quickly to avoid the stage of excitement in light narcosis, and the rest very slowly, taking 10–15 min in all). The mean ( $\pm$  S.D.) dosage used in these experiments was

Pentobarbitone	Cat,	$31.8 \pm 13.3$ mg/kg
	Dog,	$33.9 \pm 4.5$ mg/kg
Chloralose	Cat,	$97.6 \pm 13.0$ mg/kg
	Dog,	108.5 mg/kg (only used once)
Chloralose and urethane	Cat,	$64.4 \pm 9.9$ and $64.4 \pm 9.9$ mg/kg
	Dog,	$59.1 \pm 6.0$ and $59.1 \pm 6.0$ mg/kg

*General Preparation* —After anaesthetisation, the animal was suspended in a canvas sling by which it was partially submerged in a bath of Ringer solution at  $37^{\circ}\text{C}$ , and tracheal and venous cannulae were then inserted. Under these conditions, the majority of animals that were good surgical risks lived 9 hours, and several had to be destroyed after surviving 12 hours. Further preparation depended upon the type of experiment to be made, but the long survival time of the animals frequently allowed more than one type to be carried out. Four methods of study were used, but before these can be described some points of anatomy must be mentioned,

*Anatomy* —In the absence of a detailed account in the literature of the blood and nerve supply to the mammary glands of the dog and cat, a short preliminary study of this subject was made. The nerve supply was worked out by the dissection of living or recently killed animals. The course and distribution of the mammary blood-vessels was followed by studying preparations injected with coloured suspensions of Neoprene latex, according to the technique of Trueta, Barclay, Franklin, Daniel and Prichard [1947]. The only points emerging from this study which need emphasis here are the following

(a) In the lactating animal, in which the formerly separate glands have merged during pregnancy into two confluent longitudinal bands of tissue (hereafter referred to according to the numbers indicated in fig. 1a, R and L 1 to 4), the mammary glands receive two main blood supplies, anteriorly from the internal thoracic (internal mammary), and posteriorly from the subcutaneous abdominal (posterior mammary [Turner, 1939]) vessels. The latter are the anterior continuations of the external pudic vessels after they have passed through the inguinal canal.

(see fig 1a) After the administration of heparin (1000 I U per kg), the record of the blood pressure in a carotid artery was started. The left femoral artery and vein were then cannulated for the respective purposes of supplying blood to the pump reservoir (R) and receiving it back again from the perfused gland. The reservoir and pump were then filled with blood from the animal, whilst blood or 6.5 per cent gum Ringer was injected intravenously to maintain the blood volume. Heparin (100 I U) was placed in the reservoir before filling, and liquid paraffin was used to diminish the loss of gases from the surface of the blood. The subcutaneous abdominal vessels were then cannulated, care being taken not to ligate the lymphatics, which otherwise became quickly distended. During this procedure the anastomotic vessels were still intact, but immediately afterwards they were quickly ligated and severed and the gland transferred to the gland chamber. The cannulated artery was connected to the pump and perfusion begun. The period during which the tissue was completely without a blood supply, that is before the circulation was re-established, was 3 to 5 minutes. At the outset the stroke volume of the pump was minimal, and it was gradually increased until the perfusion pressure was about 120 mm Hg. For 15 to 20 minutes thereafter the organ was left undisturbed in its warm chamber, and was kept moist throughout the experiment by a covering of cotton-wool saturated with Ringer solution. In a few experiments glands 3 and 4 were perfused together in this way, since the blood-supply to gland 3 from the subcutaneous abdominal artery is sufficient for this purpose.

*Sources of Error*—Certain irregularities in the pressure tracing were found to be related to artefacts arising from the method itself. The factors thought to be concerned were

(a) *Fibrin Formation*—This was frequently found upon microscopic examination of the blood in the perfusion system in early cat experiments, in spite of the liberal use of heparin, up to a dosage of 2400 I U per kg. The tendency to form these minute fibrin clots may have been increased by the slow flow of blood through the pump (completely changed only once every 20–30 min), but was not encountered in the dog, in which the same conditions prevailed. However, in confirmation of the work of Bjork [1948], the treatment of the glassware with an organic silicone (methylchlorosilane, General Electric Dri-film No 9987, or "Teddol," British Thompson Houston Co, as a 5 per cent solution in carbon tetrachloride), and the use of synthetic plastic tubing (Portex or Polythene) instead of rubber, greatly lessened the number of clots formed. In two experiments tests were made which indicate that the danger of clots can also be reduced if a large proportion of the circulating blood is defibrinated before adding heparin. For this purpose 50 c.c. samples of arterial blood were repeatedly withdrawn from the cat, defibrinated and returned, until 300 c.c. had thus been treated and the

Massart, 1947], in which it is difficult to provide, in the correct quantities, the milk precursors and hormones, etc required by the active mammary gland. Since vasoconstriction occurred in the gland as a result of the trauma associated with dissection, it seemed preferable to use a pump giving nearly constant volume output over a wide range of pressures. For this purpose a Dale-Schuster pump with valves and pump chamber of reduced size was employed. It had a capacity of about 30 cc (including connections), and could be readily adjusted to deliver less

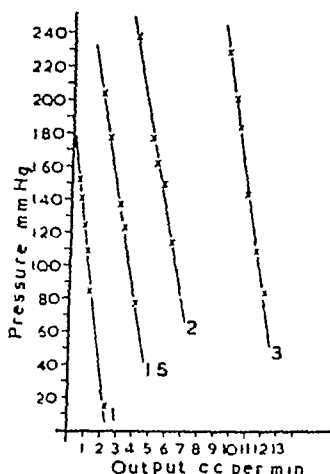


FIG. 1b —Calibration of the perfusion apparatus used, showing the fall in output with the rise in peripheral resistance. The arabic numerals by each graph refer to the stroke calibrations on the Dale Schuster pump.

than 1 cc of blood per min. There was the disadvantage that, as is shown in fig. 1b, the output diminished by 0.2 cc per 10 mm Hg rise in pressure. In this, however, it was more efficient than the other constant volume output pumps, designed for a similar purpose, which were tested. Thus one of a similar design to that used by Gaddum, Peart and Vogt [1949] showed a fall in output of 0.4 cc for each 10 mm Hg rise in pressure, while a simple pump activated by a piston made from a ground-glass syringe [see Brodie, 1903] was even less efficient. The effect of this factor could be assessed in the analysis of the experiments by reference to the calibration shown in fig. 1b, or by the simultaneous measurement of the venous outflow during perfusion.

The further preparation of the animal for perfusion consisted of dissecting one of the inguinal mammary glands (R or L 4) free from all its surrounding structures, including skin, and leaving it in contact with the animal by only the subcutaneous abdominal vessels and lymphatics, and the anastomotic vessels reaching it through the penultimate gland 3

(vasoconstriction to adrenaline and the ejection of milk with oxytocin) were still obtained

Some milk secretion apparently continued during perfusion. This was suggested by the fact that if all the preformed milk was expelled from the ducts and alveoli with oxytocin at the beginning of perfusion, more milk was ejected from the gland if oxytocin were given again after a sufficiently long interval (1 hour). It is well known that oxytocin has no action on the flow of milk if repeated soon after a first dose, because there is little or no milk left to be ejected. The positive response after 1 hour indicated the probability that some milk must have been secreted during this time.

Macroscopic and microscopic examination of the perfused glands was carried out using the opposite unperfused glands as controls. In general, the only pathological lesions demonstrable by a variety of staining methods were interlobular oedema and distension of the perivascular lymphatics, particularly if the main efferent lymphatics were accidentally tied during the cannulation of the subcutaneous abdominal vessels. Sections from glands, which for a variety of reasons (usually fibrin embolism) were perfusion failures, showed in addition interalveolar congestion and even hæmorrhage.

The chief criticism of the method was that the blood flow through the organ was probably low. This was suggested by the following considerations:

(1) Cannulation of either the main artery or vein to the mammary glands appeared to decrease the blood flow, irrespective of whether this entailed perfusion. Thus the blood-flow through the perfused glands in these experiments was  $0.036 \pm 0.012$  c.c./g./min. in the dog and  $0.025 \pm 0.01$  in the cat. A similar low figure ( $0.02 \pm 0.01$ ) was obtained when the venous outflow of the gland *in situ* in the cat was measured directly from a cannula in the vein. However, indirect blood flow measurement in the cat by plethysmography (clamping the vein for 5 sec. and measuring the increase in the volume of the gland) gave much higher figures ( $0.195 \pm 0.16$ ). These values may be compared with those of Jung [1932 a, b, 1933], who measured the mammary blood flow in two lactating goats with a Ludwig stromuhr, using ethyl chloride local anæsthesia. It is probable that the restraint involved in arterial cannulation in a conscious animal was partly responsible for the intermediate values obtained ( $0.055-0.072$  c.c./g./min.).

(2) The number of capillaries open at the end of perfusion was determined by injecting a small quantity of India ink intra-arterially immediately before stopping the pump, or by staining sections by Pickworth's [1934] benzidine method. Comparison with sections from glands that had been totally injected with India ink revealed that only about half the total number of capillaries were open.

(3) The amount of blood left in the organ at the end of perfusion

blood no longer clotted. However, in the majority of experiments the use of silicined glassware and plastic tubing rendered this lengthy procedure unnecessary.

(b) *Temperature*—In the first apparatus constructed for these experiments, although the isolated mammary gland and the perfusion pump were each contained in warmed chambers, the latter were separated by a short distance over which a slight drop in temperature of the perfusate occurred, depending upon the room temperature (see fig 1a). When an improved water bath, which held both the perfusion pump and the gland chamber, was substituted for the old arrangement, perfusion pressure irregularities were greatly reduced in number and extent. Fig 7 is representative of earlier experiments in which the temperature of the perfusate was inadequately controlled and pressure irregularities occurred in consequence (compare with fig 6). The great sensitivity of the mammary blood vessels to cold was further confirmed in one experiment on a cat, in which the water was let out of the improved water bath during perfusion and replaced by cold. As the temperature in the gland chamber fell to  $25^{\circ}\text{C}$ , the perfusion pressure steadily rose from 62 to 87 mm Hg (43.2 per cent), and the venous outflow decreased from 0.67 to 0.28 c.c./min (58.2 per cent), and some pressure irregularities were also seen. Upon replacing the cold water by water at  $39^{\circ}\text{C}$  these changes were reversed, the perfusion pressure falling to 50 mm Hg and the venous outflow increasing to 0.9 c.c./min.

In addition to these two factors there was the possibility that a high blood sedimentation rate, which was noted in many cat experiments, might account for some of the irregularities in pressure, but this was excluded, because of the lack of any correlation between their occurrence and a high B.S.R. Further, by the addition of a shunt to the outflow of the pump (screw clip A in fig 1a closed and B adjusted to bring the P.P. to its previous value) it was possible to show that the irregularities were not related to mechanical errors in the pump itself. Finally, it should be mentioned that with the very small gland of the cat, great care was needed in adjusting the position of the cannulae to avoid uncontrolled changes due to partial mechanical stricture of the supplying vessels.

*General Results of Perfusion*—Depending upon the survival of the animal, perfusion was continued for 1 to 6 hours, the tissue being considered "alive" as long as responses could still be obtained, and the arterio-venous difference in the colour of the blood gave evidence of active tissue respiration. The experiments were frequently stopped because of the death of the animal, at which stage the perfused gland was still viable on the above criteria. In one experiment on a dog, after perfusing for 6.3 hours using an artificial oxygenator (the dog having died after a perfusion of 2 hours), the gland was stored overnight at  $-4^{\circ}\text{C}$ , and upon perfusing again 15 hours later, responses to drugs



through its convex side. A 100-W bulb was sufficient for magnifications up to 50 times, but a 750-W Photoflood bulb No 1 was used for photography.

### General

*Nerve Stimulation*—This was carried out in the majority of experiments with the Ritchie-Sneath square wave stimulator, Multitone [Ritchie, 1944], with which one can independently vary the voltage, frequency and pulse length. An induction coil was occasionally used for comparison with the electronic stimulator.

*Drugs*—These were made up in Ringer or normal saline, and when injected into the gland intra-arterially, the volume of fluid was kept at 0.1 c.c. The following substances were used: Heparin (Liquemin Roche, 1000 International Units per c.c.), Adrenalin chloride (P. D. & Co.), Adrenalina (B. D. H.), Acetylcholine chloride (Roche), Cocaine (B. D. H.), Procaine hydrochloride 1 per cent (Bayer), Atropine (B. D. H.), Eserine sulphate (B. D. H.), Nicotine (B. D. H.), Histamine acid phosphate (B. D. H.), Pitressin and Pitocin (P. D. & Co.), Ergotamine ethanesulphonate (B. D. H.), Ergotamine tartrate and Dihydroergotamine (Sandoz).

### RESULTS

Stimulation of the distal cut end of the external spermatic nerve usually caused vasoconstriction (see Table I). In the case of the intact mammary gland *in situ* this was evidenced by a diminution in gland volume of 5.3 per cent or less (average about 1 per cent), persisting for 0.5 to 7 minutes, together with a simultaneous decrease in the venous outflow of 10–100 per cent (figs 2 and 3). These responses were observed in both dogs and cats and were unaccompanied by any change

TABLE I—RESPONSES TO NERVE STIMULATION

Numbers of positive responses to stimulation of various spinal nerves

Type of experiment	Species	Vasoconstriction		Vasodilatation	
Perfusion	Cat	115	(16)	2	(1)
	Dog	80	(5)		
Plethysmography	Cat	171	(18)	14	(2)
	Dog	13	(1)		
Blood flow measurement	Cat	16	(5)		
Microscopy	Cat	50	(7)		
Total	Cat	352		16	
	Dog	93			

The numbers in brackets refer to the number of experiments involved

was determined by washing through the gland with a known amount of saline and comparing photoelectrically the amount of hæmoglobin collected in the saline with that in an equal volume of saline to which a known amount of the same blood had been added. It was found to be only about 30 per cent of the total capacity of the gland, as estimated by the volume of a complete Neoprene cast of the vessels (made at 200 mm Hg injection pressure)

### *Plethysmography and Blood-flow Measurements*

These experiments were confined to a study of glands 4 or 3 and 4 together, which were completely isolated from all surrounding structures and left connected to the cat by only the subcutaneous abdominal vessels and lymphatics. The separated tissue was then enclosed in a plethysmograph of 30–40 c.c. capacity for gland volume recording, and this was connected to a Brodie volume recorder (3 c.c. capacity). It was possible to record gland volume changes of 0.01–0.02 c.c. by using a smaller version of the free swing lever described by Afford [1948], weighing only 100 mg. The blood flow was measured by cannulating the subcutaneous abdominal vein and recording the venous outflow with a drop recorder (designed by Professor A. E. Ritchie). In both types of experiments the general systemic blood pressure was recorded with a mercury or small membrane manometer.

When attempts were made to record mammary gland volume and the venous outflow simultaneously, it was found that this was unsuccessful because of the passive shrinkage in gland volume which occurred when the subcutaneous abdominal vein was opened for cannulation (venous pressure about 4–5 cm. blood). Presumably there was then less blood in the organ to be squeezed out by an active vasoconstriction, and so the response to nerve stimulation was smaller than when the vein was intact. Recently it was found in one experiment that if the animal were placed prone instead of supine, so that the gland hung down below the abdomen in approximately the normal position, opening the vein did not induce this passive shrinkage of the gland, and the vasoconstrictor effect of nerve stimulation could still be effectively demonstrated.

### *The Microscopical Examination of Living Blood Vessels*

For these experiments the tissue was quickly exposed with a minimum of dissection, and prevented from drying and cooling by a constant stream of 1 per cent gelatin in Ringer at 38.5° C. [see Zweifach, 1948]. Indirect cool illumination was used after the quartz rod technique of Knisely [1934, 1936], but using instead a polished Perspex rod ( $\frac{1}{8}$  inch diam.) opposite a hole of similar size in the lamp house. The rod was cut at 45° at the tissue end to focus the light, which was thus reflected

in general blood pressure Results from perfusion experiments confirmed these findings Thus nerve stimulation produced a rise in perfusion pressure of up to 37 per cent of the mean resting value (average about 9 per cent) in cats, and up to 59 per cent (average about 20 per cent) in dogs, lasting 0.5 to 4 minutes In both species there was also a decrease in the venous outflow, an observation indicating that the rise

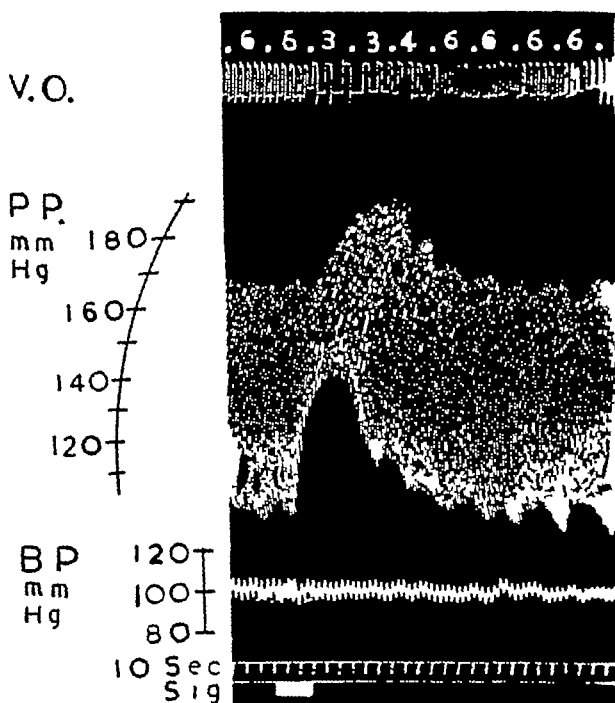


FIG 4—Expt C 49 Cat 2.8 kg Lactating 13 days Chloralose and urethane anaesthesia Perfusion of R 4 started at 2.50 p.m. Defibrinated heparinised blood V.O 17 drops per c.c. B.P by mercury manometer 3.21 p.m Stimulation of external spermatic nerve, 0.5/50/10/28

in pressure itself did not fully reflect the intensity of the vasoconstriction produced (figs 4, 6 and 7) The responses were obtained at all levels of the resting perfusion pressure, and also at the height of a pressor response to a moderate dose of adrenaline In the dog there was frequently a temporary increase in the venous outflow, occurring at the time when the perfusion pressure was rising, which was followed by the usual decrease (fig 7) This was not seen in cat perfusions, but was observed in one cat experiment in which the venous outflow was recorded from the intact gland In three cat perfusion experiments the pressure curve to nerve stimulation showed a flattening or even a second rise succeeding the initial peak

In all types of experiment the size and duration of response could be

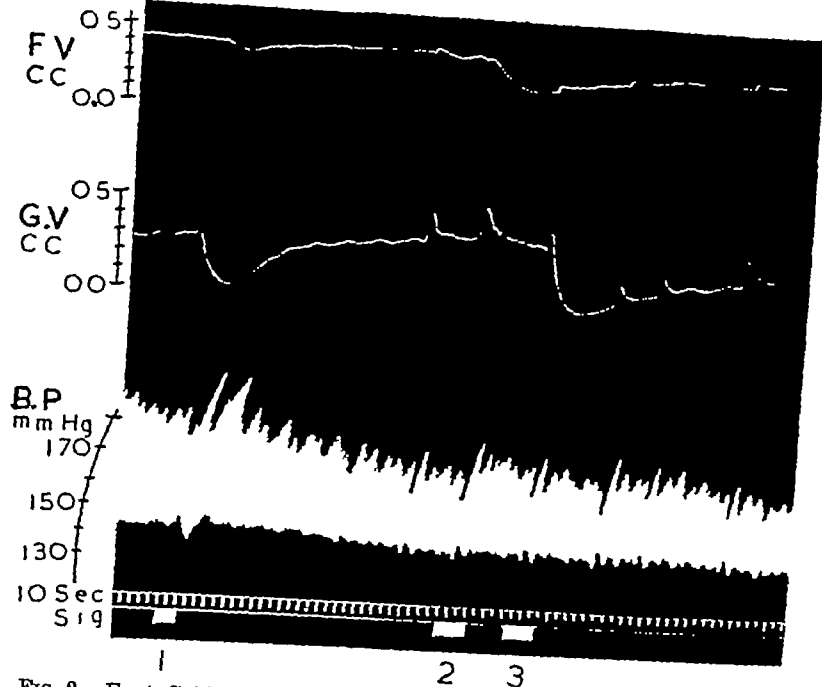


Fig 2—Expt C 39 Cat 28 kg Lactating 3 days Pentobarbitone anaesthesia Left inguinal fat and R 4 in plethysmographs at 11 10 and 1 p.m. respectively, with circulation intact Fat volume (FV) 11 cc Gland volume (GV) 19.5 cc BP by membrane manometer Cat supine

1	3 32 p.m.	Adrenaline 1 $\mu$ g, intravenously		
2	3 37 "	Stimulation of external spermatic nerve, 10/50/1/30	Left side	
3	3 38 "	" " " " "	Right side	

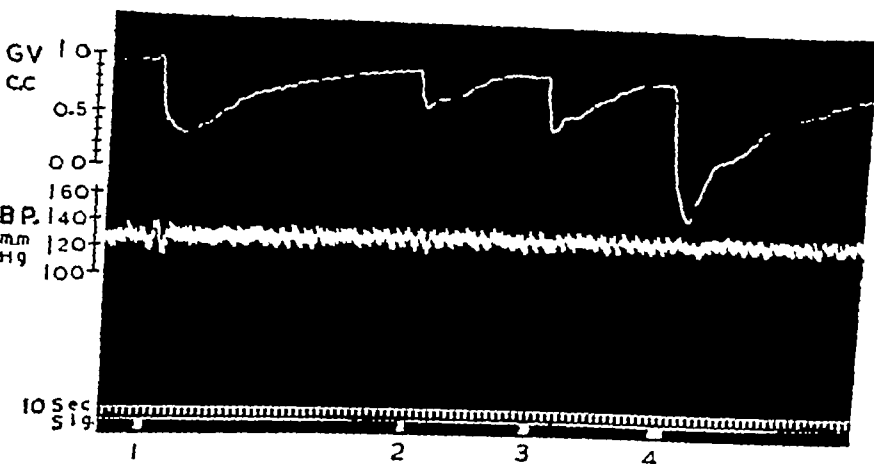
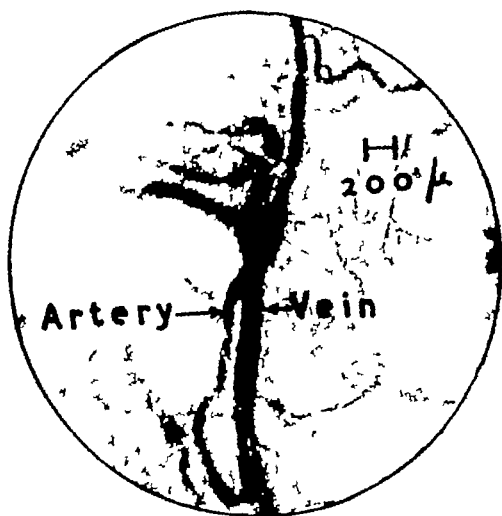


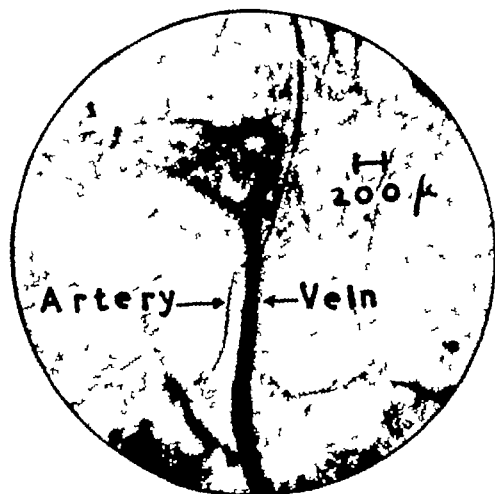
Fig 3—Expt D 1 Dog 17.2 kg 4 days involution Pentobarbitone anaesthesia. L 5 in plethysmograph at 12 31 p.m., with circulation intact Gland volume (GV) 90 cc BP by mercury manometer Dog supine

1	1 53 p.m.	Adrenaline 2 $\mu$ g, intravenously		
2	1 59 "	Stimulation of external spermatic nerve	10/5/1/5	
3	2 02 "	" " " "	10/5/1/10	
4	2 05 "	" " " "	10/5/1/15	

N.B.—These responses were not conditioned by the previous dose of adrenaline



1



2

FIG 5—Expt C 68 Cat 2.8 kg Lactating 24 days Chloralose and urethane anaesthesia Photomicrographs of the living vessels on the surface of gland R 4

- |   |          |  |
|---|----------|--|
| 1 | 2 02 p m | Small artery and vein (15 sec exposure)  |
| 2 | 2 04 "   | Same vessels 15 sec after stimulation of external spermatic nerve (10/50/1/10) Same exposure |

increased by lengthening the time of stimulation (up to a maximum of about 15 sec) (fig 3), and to a more variable extent by increasing the voltage (up to a maximum of 0.5–10 V), pulse length (up to 1–10 msec) and the frequency (up to 5–50/sec). A maximal response could invariably be obtained by a stimulus of 10 volts, 50 stimuli/sec, each of 1 msec lasting 15 sec (signified in this paper 10/50/1/15).

In addition to the external spermatic nerve, the mixed spinal nerves thoracic 13 to lumbar 4, supplying mammary glands 3 and 4, were also found to include vasoconstrictor fibres. The inguinal glands 4, which have been given the most attention in the present study, receive fibres from cutaneous lumbar nerves 2, 3 and 4, but most of their innervation from the external spermatic nerve, which also arises from lumbar 4. The peripheral distribution has not been worked out in detail, but from microscopical examination it appeared that there is considerable overlap in the areas innervated by adjacent nerves.

These results were not in themselves conclusive proof for the existence of vasoconstrictor nerve fibres to the mammary glands as such, because they referred to mammary tissue (inguinal gland 10–20 g), plus 1–2 g (5–10 per cent by weight) of adipose tissue and the superficial inguinal lymph glands (about 300 mg). The fat and lymph glands were the tissues that it was not practicable to separate from the mammary glands because they were so intimately related to the branches of vessels and nerves entering the gland. Possibly the responses recorded represented the activity of vasomotor nerves to the large vessels outside the gland and to the fat between them. Indeed, on actual test, it was shown that the volume of the inguinal fat, when measured separately, shrank on nerve stimulation (fig 2), thus providing further evidence for the existence of vasomotor fibres in adipose tissue [see Wertheimer and Shapiro, 1948]. However, in four experiments, in which the mammary gland was completely separated from all fat and lymph tissue, it too showed positive responses to nerve stimulation (fig 2). The decrease in gland volume then recorded was usually about equal to the volume of a Neoprene cast of all the large vessels outside the gland proper, and since these vessels contracted only very slightly during nerve stimulation, it was concluded that the smaller vessels within the gland were mainly responsible for the responses recorded.

To be quite certain that the deductions from these experiments were correct, microscopic examination of the living vessels on or near the surface of the mammary glands was carried out. This revealed that nerve stimulation produced visible constriction of small arteries (20–100  $\mu$ ), but little if any change in the calibre of the accompanying vein (50–150  $\mu$ ) and capillaries (fig 5), although the application of a small dose of adrenaline to the capillaries on the surface of a lobule did produce blanching. To nerve stimulation (10/50/1/15) there was a latent period of about 2 seconds, after which the artery rapidly contracted

and the blood flow in the artery and vein slowed or even stopped. During the response, which lasted 1 to 2 minutes, the blood corpuscles in the vein could be seen proceeding in a series of jerks, and even going backwards on occasions. Serial sections of such a pair of vessels that had been observed in the living state (artery  $50\ \mu$  and vein  $100\ \mu$ ) showed that smooth muscle could only be detected in the medial coat of the artery and that three very fine nerve fibres ran between its media and adventitia.

The vasoconstriction produced by nerve stimulation was shown to be unaffected by eserine sulphate (0.1 mg/kg into the whole animal or  $50\text{--}100\ \mu\text{g}$  intra-arterially), by atropine sulphate (0.3 mg/kg intravenously or  $50\text{--}100\ \mu\text{g}$  intra-arterially) and by nicotine (0.5 mg intra-arterially), but was abolished by ergotoxine, ergotamine and dihydroergotamine (0.3 mg/kg intravenously or 0.1–1.0 mg intra-arterially) (figs 6 and 9). Reversal of the response with ergot preparations was seen in only 7 out of 14 experiments, and in these the vasodilatation produced by nerve stimulation was small and transient. In four perfusion experiments (3 cats and 1 dog) dihydroergotamine, in addition to abolishing the effects of nerve stimulation, produced a marked and lasting vasodilatation, as shown by a permanent fall in perfusion pressure and a rise in the venous outflow. Cocaine hydrochloride was administered intravenously to the whole cat according to the method of Rosenblueth and Schlossberg [1931], and, in conformity with their findings, potentiated (50–100 per cent) and prolonged (up to 10 times) the vasoconstrictor response to nerve stimulation, but only when sufficient had been injected to produce toxic effects on the heart. Tannic acid (0.5 mg intravenously and  $10\ \mu\text{g}$  intra-arterially) also potentiated (20–100 per cent) and prolonged (30–110 per cent) the responses to nerve stimulation in 6 out of 8 experiments in the dog and cat (plethysmography and perfusion). This was of interest in view of the recent finding of Konzett [1948] that this drug has the same effects on the physiological actions of adrenaline, an action also found in these experiments. These findings showed that the nerve endings concerned were adrenergic.

*Reflex Vasoconstriction*—This was demonstrated in the cat by comparing the responses of the intact gland with that of its opposite control, which was denervated. In this preparation, anoxia, produced by clamping the trachea or by rebreathing nitrogen, caused a passive increase in the volume of the denervated gland coinciding with the rise in B.P., but a decrease in the volume of the innervated side. When the latter was also denervated a passive response to anoxia was then seen in both glands. The vasoconstriction of the innervated gland, which must have been central or reflex in origin, was of the same magnitude as was subsequently produced when the distal cut end of the same nerve was maximally stimulated.





no greater tendency for vasodilator responses to appear when the vessels were in a more constricted state, as judged by a high resting perfusion pressure or a poor blood flow through the intact gland. In one experiment, weak electrical stimuli which regularly produced a diminution in gland volume had the opposite effect after tannic acid administration.

### *The Motor Actions of Drugs on the Mammary Blood Vessels*

Table II summarises the results obtained, and particular reference will be made to only adrenaline and acetylcholine.

**Adrenaline**—The sensitivity to this substance was great. By intra-arterial injection into the perfused gland the threshold dose varied between 0.05 and 0.005  $\mu\text{g}$  in the cat and 0.001 and 0.0001  $\mu\text{g}$  in the dog. Since the glands weighed 10–20 g in the cat and 40–90 g in the dog, it appeared that the dog's mammary vessels were more sensitive

TABLE II—RESPONSES TO DRUGS

Substance	Species	Response	Minimum effective dose used	
			Whole animal	Isolated gland.
Adrenaline	Cat	Vasoconstriction	0.01 $\mu\text{g}$ *	0.005 $\mu\text{g}$ *
	Dog	"	1.0 $\mu\text{g}$	0.0001 $\mu\text{g}$ *
Acetylcholine	Cat	Vasodilatation	1.0 $\mu\text{g}$	0.2 $\mu\text{g}$ *
	Dog	"	1.0 $\mu\text{g}$	0.1 $\mu\text{g}$
Histamine	Cat	Vasodilatation	0.1 $\mu\text{g}$	0.1 $\mu\text{g}$
	Dog	"		0.1 $\mu\text{g}$
Urethane	Cat	Vasodilatation	10 mg	0.3 mg
	Dog	"		1.0 mg *
Pentobarbitone	Cat	Vasodilatation		0.05 mg
	Dog	"		0.01 mg
Chloralose	Cat	Vasoconstriction		1.0 mg *
	Dog	"		1.0 mg *
Pitocin (oxytocin)	Cat	Usually a vasoconstriction, sometimes vasodilatation with smallest doses	0.01 unit *	0.001 unit *
	Dog			0.0001 unit *
	Cat	Ejection of milk	0.01 unit *	0.01 unit *
	Dog	"		0.001 unit *
Pitressin	Dog	Vasoconstriction, no ejection		0.01 m. unit.
Pilocarpine	Cat	Vasodilatation	0.5 mg	
Eserine	Cat	Vasoconstriction		50 $\mu\text{g}$
	Dog	"		50 $\mu\text{g}$
Atropine	Cat	Varied		50 $\mu\text{g}$
	Dog	"		50 $\mu\text{g}$
Nicotine	Cat	Vasodilatation		100 $\mu\text{g}$
	Dog	"		10 mg (only once)
Dihydroergotamine	Cat	Varied	0.5 mg	100 $\mu\text{g}$
	Dog	"	1.0 mg	100 $\mu\text{g}$

Only those figures marked with an asterisk \* represent threshold doses.

*Vasodilatation*—Nerve stimulation occasionally produced small vasodilator responses in the cat (Table I), usually with weak stimuli

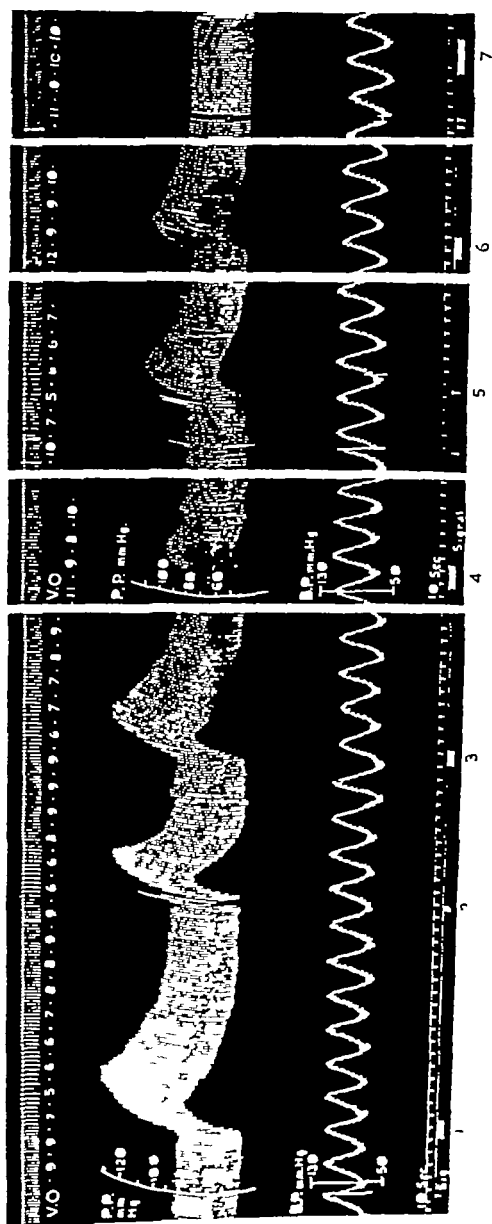


FIG 6.—Expt D 4 Dog 23.1 kg 7 days' involution Chloralose and urethane anaesthesia Perfusion of R 5 started at 3.13 p.m. VO 14 drops per cc B.P. by mercury manometer

1	5.01 p.m.	Stimulation of external spermatic nerve, 10/50/1/12
2	5.03 "	Eserine sulphate 100 µg, intra arterially
3	5.06 "	Stimulation repeated, 10/50/1/12
4	5.08 "	" " " "
5	5.15 "	Atropine sulphate 100 µg, intra arterially
6	5.18 "	Stimulation repeated, 10/50/1/17
7	5.24 "	Control Nerve pulled over electrodes with no current flowing

N.B.—Note the Traube-Hering type waves in the B.P. tracing

(0.5–1.0 volt, 0.1–0.01 msec) In many other experiments such weak stimuli produced either no effect at all or vasoconstriction. There was

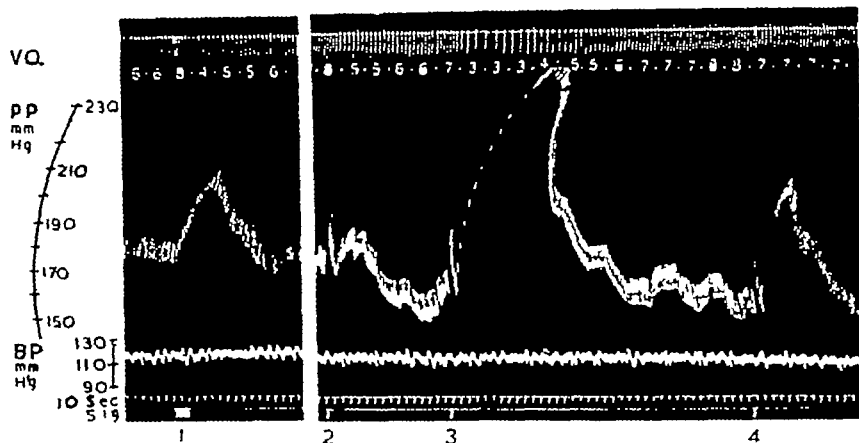


Fig 7—Expt D 3 Dog 14.5 kg 2 days' involution Chloralose anaesthesia. Perfusion of R 4 started at 2.51 p.m. VQ 14 drops per c.c. BP by mercury manometer

1	4.22 p.m.	Stimulation of external spermatic nerve, 10/50/1/17
2	4.32 "	Adrenaline 0.001 $\mu$ g, intravenously
3	4.35 "	" 0.1 $\mu$ g, "
4	4.41 "	" 0.01 $\mu$ g, "

See also fig 8

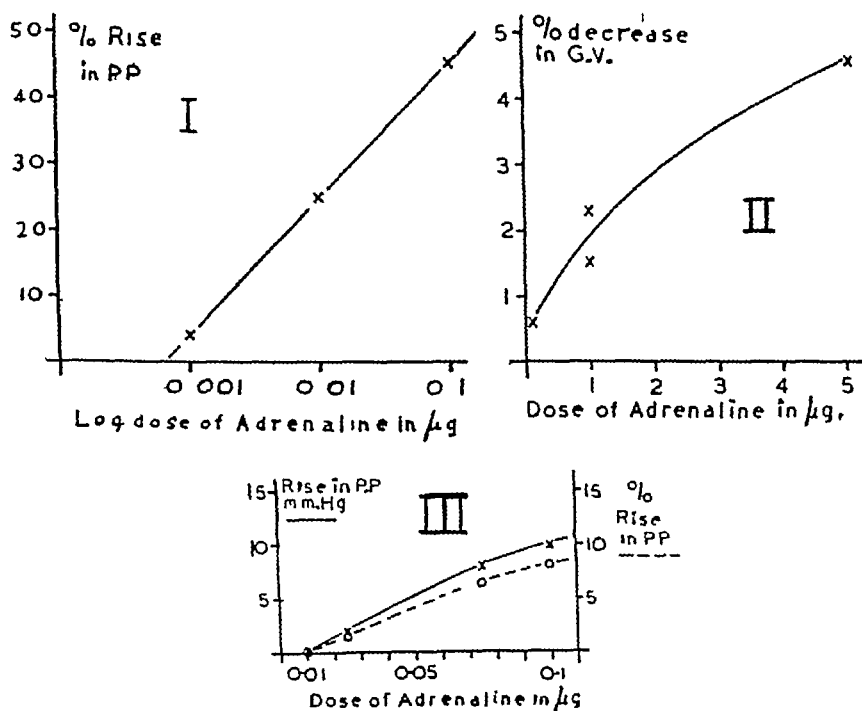


Fig 8—Dose response curves to adrenaline administration

- I Expt D 3 Dog 14.5 kg Perfusion Taken from fig 7
- II Expt C 39 Cat 2.8 kg Intact gland in plethysmograph Adrenaline by intravenous injection. Same experiment as figs 2 and 9
- III Expt. C 16 Cat 3.8 kg Lactating 5 days Pentobarbitone and urothane anaesthesia. L 4 perfused for 1 hour

to adrenaline than were the cat's. The duration of the response in both species varied between 1 and 20 minutes according to the dose (see fig 7). The smallest amounts occasionally caused a fall in perfusion pressure, which was often followed by the usual pressor response. Diphasic responses similar to those described as occasional effects of nerve stimulation were also observed a few times with adrenaline. By intravenous injection into the whole animal, the threshold dose that produced a shrinkage in mammary gland volume accompanied by a slight rise or no change in B P was  $0.01-0.1 \mu\text{g}$  in the cat (fig 2). Adrenaline was tested in this way in only two dogs (11.6 and 17.3 kg),  $1 \mu\text{g}$  produced vasoconstriction in each case (fig 3). In all experiments the size of response varied with the dosage. Typical dose-response curves are shown in fig 8. The ergot preparations listed, used in the dosage required to suppress the results of nerve stimulation, also abolished (3 experiments) or temporarily reversed (7 experiments) the effects of adrenaline (fig 9).

*Acetylcholine*—This produced vasodilatation in doses of  $0.1-1.0 \mu\text{g}$  in the isolated gland of the dog and cat (unserinised), but in the whole animal the results were more variable and the individual sensitivity to the drug covered a wide range. Small doses ( $1 \mu\text{g}$ ) usually produced a fall in B P and an increase in mammary gland volume, but a fall in the latter was also sometimes seen, which was interpreted as being due to a fall in inflow pressure. A similar variation was encountered in one experiment in which  $1 \mu\text{g}$  by intra-arterial injection either increased or decreased the venous outflow from the intact gland. The fact that this dose regularly produced vasodilatation in the perfused gland suggests that the results in the whole animal were due to capacity effects.

### *Spontaneous Gland Volume Changes*

The spontaneous pressure changes met with in early perfusion experiments have been discussed under Methods, where it was suggested that they were largely due to artefacts arising from imperfections in technique. However, in the plethysmography experiments, a greater or lesser amount of spontaneous variation in mammary gland volume was seen in 20 out of 21 experiments in the cat and in 1 out of 2 dogs. Although these variations did not preclude the recording and interpretation of responses produced experimentally, an attempt was made to determine their origin and cause.

In addition to pulse and respiratory waves, which were not always shown on the volume records, two types of spontaneous wave could be distinguished. Type I consisted of large, sudden, regular increases in gland volume, which subsided quickly. Each response lasted 20-60 seconds. They were usually only seen for limited periods in any one experiment (figs 2 and 9). Type II had a similar wave length, but were

in the B P, the general level of which fell as the bladder filled (fig 9) Opening the abdomen partially and puncturing the bladder completely reversed the fall in B P and likewise abolished the spontaneous volume changes They could be reproduced, however, by gently pressing on the abdomen before and on the posterior vena cava after relieving the distended bladder (fig 9) They also reappeared spontaneously when the bladder was refilled with saline to its original state of tension Thus it was concluded that, in this experiment at least, the spontaneous changes in B P, fat volume and mammary gland volume were due to pressure on the vena cava, damming blood up peripherally and decreasing the venous return to the heart In this particular case it appeared that the efforts of the bladder to evacuate its contents were the motive force, but in other experiments it appeared to be either bladder, uterine or rectal movements Such effects were only entirely abolished by complete evisceration, thus stressing the fact that any slight pressure on the abdominal veins can cause such phenomena

When the venous pressure was recorded as well as the mammary gland volume, it was confirmed that type I waves were accompanied by a rise in venous pressure (fig 10), but that the smaller spontaneous waves called type II were independent of venous pressure variations, both when it was measured in the femoral vein and in the subcutaneous abdominal vein itself (fig 10) Type II waves were also seen in other experiments, in which the abdomen was open and the bladder empty, and occurred in the mammary gland on one side of the cat when there was no such change in the inguinal fat on the other The recent finding of Peeters, Coussens and Oyeart [1949], Peeters, Coussens and Sierens [1949], and Peeters, Massart, Oyeart and Coussens [1948] that the cow's teats undergo rhythmical contractions under certain conditions, suggested that this factor might be concerned here However, removal of the teat and areola had no effect upon the spontaneous volume changes, and since these tissues weigh only about 100 mg in the cat, it is probable that if teat movements were taking place they would be too small to be recorded by the apparatus used Another explanation might have been that uterine movements produced the small waves of type II by pulling on the round ligament and thus compressing the external pudic vein as it passed through the inguinal canal However, removal of the round ligament had no effect on the gland volume changes Histological examination revealed a few scattered smooth muscle cells in the interlobular connective tissue of the gland, and it was found that some bundles of striated muscle from the panniculus carnosus muscle traverse the glands (even penetrating into the teat in one case), but it was not considered that either type of muscle could easily produce the mammary gland volume changes in question

Finally, it was discovered that type II waves frequently occurred simultaneously in both inguinal mammary glands (L and R 4 in fig 1a)

smaller and less regular. It was difficult to decide whether the primary change was a contraction or dilatation of the gland. They were usually present throughout an experiment, but tended to disappear as the gland deteriorated in its responses to nerve stimulation and drugs (figs 10 and 11).

Type I waves were usually accompanied by a slight fall in B P, and were unaffected by cutting the external spermatic nerve, by bilateral

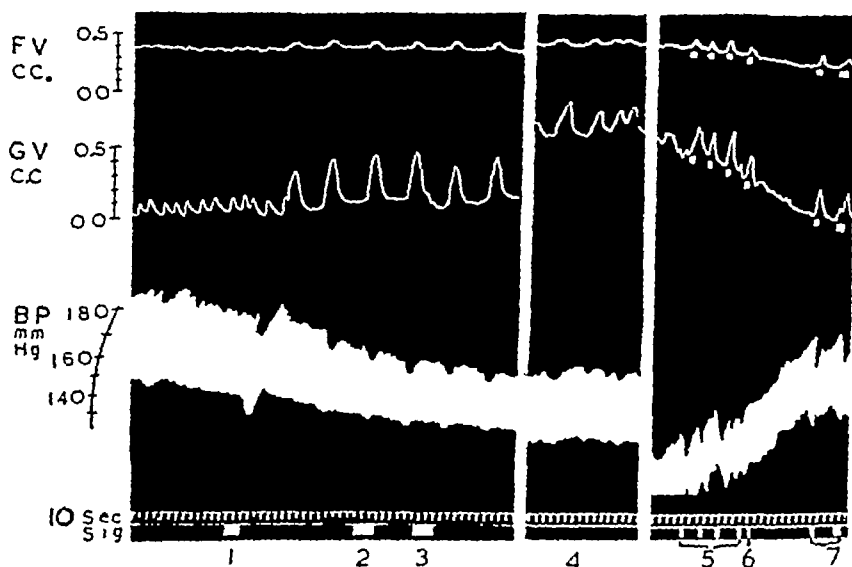


Fig 9—Expt C 39 Cat Same experiment as fig 2 Spontaneous changes in fat volume, mammary gland volume and B P

4 17 p.m	Dihydroergotamine 1 mg, intravenously	
1 4 26 "	Adrenaline 10 µg, intravenously	
2 4 29 "	Stimulation of external spermatic nerve, 10/50/1/25	Left side
3 4 30 "	" " "	Right side
4 5 35 "	Continued spontaneous responses	
5 6 14 "	Abdomen opened and the very full bladder pressed four times	
6 6 15 "	Bladder punctured	
7 6 17 "	Posterior vena cava pressed gently twice	

vagotomy, by bilateral adrenalectomy, or by clamping the carotid arteries. They were also unaffected by liberal applications of procaine, cocaine, phenol and formalin to the subcutaneous abdominal vessels and lymphatics (the only connections with the cat). They were sometimes caused by moving the rectal thermometer, again after thorough denervation. In yet another experiment spontaneous increases of this type in the volume of the inguinal fat on one side, and the inguinal mammary gland on the other (both denervated), steadily increased in size as the urinary bladder became more and more distended during the experiment. These changes were accompanied by opposing changes

and in a kidney, and usually bore a simple relationship to Traube-Hering type waves in the blood-pressure tracing (one gland volume wave corresponding to 1 or 2 B P waves) These B P waves were similar to those described by Barcroft and Nisimaru [1932] and by Barcroft, Nisimaru and Steggerda [1932], which were attributed to contraction and relaxation of the spleen and intestines and were abolished by clamping the splenic and intestinal vessels These procedures, on the contrary, had no effect on the B P or organ volume waves encountered in the present experiments However, when the respiration was recorded in addition, it was seen that the waves in the B P and the mammary gland volume could be correlated with rhythmical fluctuations in the depth of respiration (fig 11),<sup>1</sup> and that they disappeared when the chest was opened and the animal put on artificial respiration (Starling Ideal Pump)

It was concluded, therefore, that neither type of spontaneous mammary gland volume changes met in these experiments was specifically concerned with the mammary glands type I were due to compression of the great veins in the abdomen by the abdominal viscera, and type II were due to changes in the blood flow through the glands, which was of central origin and probably connected with variations in the depth of respiration

### DISCUSSION

From these experiments it is apparent that the mammary glands receive vasomotor fibres from the cutaneous mixed spinal nerves The usual response to electrical stimulation of the distal cut ends of these nerves is a vasoconstriction of the small arteries and arterioles in the mammary glands, the vessels which histological examination reveals have a well formed muscular medial coat and two or three fine nerves between the media and adventitia It can also be shown that the vasoconstrictor nerves concerned are adrenergic, because (a) adrenaline has the same action, (b) the vasoconstriction is unaffected by eserine and atropine, (c) it is potentiated by cocaine and tannic acid, and (d) abolished or reversed by ergot preparations

It is well known that the mixed spinal nerves contain sympathetic vasomotor fibres There can be little doubt that the vasoconstrictor fibres in the lumbar nerves, which have been shown in these experiments to extend to the mammary glands, skin and subcutaneous fat, are sympathetic in origin and pass to the spinal nerves in the grey ram The location of the cell stations of the fibres concerned is beyond the scope of this paper, but many careful dissections have failed to reveal any connections between the abdominal sympathetic plexuses and the spinal nerves studied This confirms the finding of St Clair [1942], who noted an increase in the temperature of the cow's udder when he removed

<sup>1</sup> Investigated at the suggestion of Professor R J S McDowall

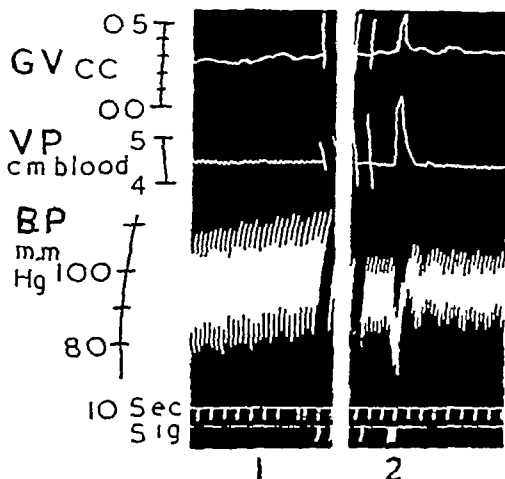


FIG 10—Expt C 52 Cat 2.2 kg Lactating 16 days Chloralose and urethane anaesthesia. R 4 in plethysmograph at 5 p.m., with circulation intact Gland volume 15 cc Venous pressure (V.P.) recorded in right subcutaneous abdominal vein B.P. by membrane manometer Cat supine

- 1 5.50 p.m. Spontaneous gland volume waves Venous pressure showing respiratory waves only
- 2 6.33 „ Gentle pressure on the abdomen, over the umbilicus

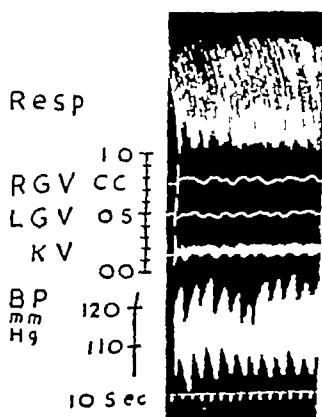


FIG 11—Expt. C 61 Cat 2.95 kg Lactating 8 days Chloralose and urethane anaesthesia Mammary glands L 4 (16 cc) R 4 (16 cc) and left kidney (K.V. 11 cc) in plethysmographs at 1.14 p.m. with circulation intact Respiration (Resp) recorded by volume recorder and a oesophageal tube ending over the base of the heart B.P. by membrane manometer Cat supine

1.45 p.m. Spontaneous changes



rat, guinea-pig and rabbit, developing in the subcutaneous fat, take over the pre-existing capillaries, which become enlarged in the region of the mammary tissue (some become differentiated into arterioles and venules), and that no new vessels are formed. It has been shown in the present experiments that the mammary fat also receives sympathetic vasoconstrictor fibres, so that the vessels taken over by the developing mammary gland are presumably already innervated, and the growth of nerves may be as limited as the growth of vessels in these species. Dabelow also states that the capillaries in the fat between the lobules, which are continuous with the vessels within the mammary lobules themselves, in the laboratory rodents, may serve as shunts for the transfer of blood from an inactive (full of milk) to an active (empty) lobule, assuming that the distended alveoli would mechanically compress the capillaries in the inactive lobules. If this hypothesis is correct, vasomotor nerves to the anastomotic vessels may be of importance for this phenomenon.

The difficulties and sources of error encountered in the methods used for these experiments need little comment. The changes in mammary gland volume produced by extrinsic factors merely emphasise the care necessary in the interpretation of plethysmograph records, and the analysis of their causes may be of wider interest in view of similar spontaneous volume waves detected by the sensitive finger plethysmographs [see Burch, 1948]. The difficulties met with in trying to perfuse the mammary glands also illustrate the great care required in discovering how far the conditions obtaining during perfusion approach the normal. It is not sufficient to assume, as has been done by other workers in this field, that the mammary tissue remains normal merely because it carries out one or more functions as in the intact unanæsthetised animal. The implications from these experiments, that the blood flow may be reduced in the perfused state on account of the vasoconstriction so easily induced by handling and cooling, shows that misleading findings may result from the use of such preparations in biochemical studies of milk secretion without some independent means of assessing their value.

#### SUMMARY

- 1 The existence of sympathetic vasoconstrictor fibres in the spinal nerves to the mammary glands of the cat and dog has been demonstrated in experiments involving blood flow measurements, plethysmography, perfusion of the isolated gland, and microscopical examination of the living vessels.

- 2 In the cat, vasoconstriction was only observed in the small arteries and arterioles, vessels which, on histological examination, were found to have much smooth muscle in the medial coat and were innervated by fine unmyelinated nerve fibres.

the inguinal nerve or the lumbar sympathetic chain, but no change when the posterior mesenteric plexus was ablated

In the ruminant the perineal nerve, which is the continuation of the pudic nerve arising from sacral nerves 2, 3 and 4, also sends fibres to the back of the mammary glands [St Clair, 1942], but this has not been found in any dissections of the mammary glands in the dog and cat. The fact that nicotine has no action upon the vasoconstriction produced by stimulating the lumbar nerves shows that there are no ganglia in the course of these nerves in the mammary glands (confirmed by a similar finding by Peeters, Coussens and Sierens [1949] in the cow and much histological work). For these reasons it is difficult to agree with the suggestion of Petersen [1942] that the mammary gland probably receives parasympathetic innervation, although the evidence on which it is based, that the gland responds to parasympathomimetic drugs, is confirmed.

No convincing evidence was found of vasodilator fibres to the mammary glands in these experiments. It may be that the small and inconstant responses observed were in fact due to the stimulation of either sympathetic or antidromic vasodilator fibres, although the latter hypothesis is unlikely, since the time relations and characteristics of the responses recorded were not typical of antidromic vasodilatation. It could be suggested that vasodilatation was not readily demonstrated because the innervated vessels were already fully dilated, but all the observations made point to the opposite conclusion. In fact, the major difficulty of the preparation for all experiments reported here was to reduce spontaneous vasoconstriction, and the conditions should have been ideal for the demonstration of vasodilatation.

Although it has not been directly shown that sympathetic vasoconstrictor fibres play any part in controlling the blood flow through the mammary glands of the unanaesthetised animal, the vasoconstriction to anoxia demonstrated in the anaesthetised state is as great as can be subsequently produced by maximal stimulation of the external spermatic nerve, and this severely curtails the blood flow through the gland for 1 to 2 minutes or more. It seems very likely, therefore, that the fall in milk yield, which is so well known to occur in the cow when subjected to fear or pain, is a direct consequence of a poor blood flow through the organ resulting from reflex vasoconstriction, and the action of adrenaline released into the circulation under these circumstances.

Vasomotor nerves have been demonstrated in these experiments only in lactation and in commencing involution. In view of the rapid growth of the mammary gland in the first half of pregnancy, and its equally rapid involution at the end of lactation, accompanied in the rabbit, according to Wahl [1915], by the formation of new vessels, it would appear that it might be a suitable tissue for following the growth and degeneration of vasomotor nerves. However, this may not be the case. Dabelow [1933] believes that the mammary glands of the mouse,

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3 The nerve endings concerned are thought to be adrenergic, because—

- (a) adrenaline has the same action in very small doses,
- (b) their effects are unaffected by eserine, atropine and nicotine,
- (c) are potentiated by cocaine and tannic acid,
- (d) abolished or reversed by ergot preparations

4 No convincing evidence has been obtained of the existence of active vasodilator fibres in the spinal nerves studied, or of parasympathetic innervation of the mammary gland

5 The mammary blood vessels are very sensitive to cooling, to which they react by vasoconstriction

6 Two types of spontaneous volume waves seen in plethysmograph experiments are described and their causes discussed

#### ACKNOWLEDGMENTS

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NOTE UPON THE STABILITY OF THE RATE OF PARA-AMINO HIPPURATE FORMATION OF RAT LIVER AND KIDNEY SLICES AND OF OXYGEN CONSUMPTION *IN VITRO* AFTER BURNING By D W VAN BEKKUM,<sup>1</sup> G H LATHE<sup>2</sup> and R A PETERS From the Department of Biochemistry, Oxford

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As the matter seemed important, we have investigated the enzymatic behaviour of liver and kidney slices during the height of the metabolic disturbance after burning (i.e. after some three days), in a fresh attempt to find some biochemical lesion related to the nitrogen loss in the urine.

The capacity of liver and kidney tissue to form para-amino hippuric acid (PAH) from para-aminobenzoic acid (PAB) and glycine has been determined as a possible index of metabolic changes related to proteins. Borsook and Dubnoff [1941] have suggested that the formation of *p*-amino hippurate (PAH) is similar to peptide bond formation taking part in protein synthesis. Recently Cohen and McGilvery [1946, 1947 *a*, 1947 *b*] have established methods for assaying the capacity of tissue slices to form PAH, and have found that PAH formation depends on the integrity of the enzyme systems concerned in liberating the energy stores of tissues.

Our experiments showed no significant change in the rate of PAH formation due to burning.

Some experiments upon  $Q_{O_2}$  in media containing various substrates were also made upon liver and kidney slices from burned rats after the three-day period. It may be recalled that Muus and Hardenburgh [1944], and Muus, Hardenburgh and Drinker [1944], found that lymph from burned limbs increased the oxygen consumption *in vitro* of liver and diaphragm of normal rats. Furthermore, within 24 hours of burning, Clark and Rossiter [1944], and Harkins and Long [1945], found no change in  $Q_{O_2}$  of slices from rabbit liver and rat liver.

#### METHOD

In the initial series of experiments upon  $Q_{PAH}$  and  $Q_{O_2}$ , 5 burned and 5 control animals (male albino rats) were studied, six liver and

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added, a "substrate effect" for both burned and unburned animals was obtained. The results, given in Table II, reveal that PAB (0.01 M), and PAB (0.01 M) plus glycine (0.1 M), significantly depress the oxygen uptake in both burned and unburned animals. In the case of glucose (0.18 M) a significant though minor reduction of  $Q_{O_2}$  was produced in

TABLE II—EFFECT OF VARIOUS SUBSTRATES ON THE  $Q_{O_2}$  OF LIVER SLICES OF BURNED AND UNBURNED RATS

(Figures in the body of the table are means and standard errors of the means  
The number of animals is in brackets)

Substrate	Change in $Q_{O_2}$ (mg wet weight) on addition of substrate		
	Burned	Unburned	Difference
Glycine	- 08 ± 07 (16)	- 10 ± 07 (14)	02 ± 1
PAB	- 16 ± 08 (16)	- 29 ± 08 (10)	13 ± 08
PAB + Glycine	- 17 ± 06 (12)	- 19 ± 07 (13)	02 ± 09
Glucose	- 09 ± 06 (16)	- 15 ± 05 (14)	06 ± 07
Lactate	+ 13 ± 05 (16)	+ 01 ± 07 (14)	12 ± 09

unburned animals by the addition. Lactate (0.35 M) significantly increased the  $Q_{O_2}$  in burned animals. In none of the five media, however, was the difference between burned and unburned animals significant.

These results indicate the remarkable fact that three days after burning, in spite of the marked disturbance of nitrogen metabolism in burned animals, so far as the liver and kidney are concerned they do not deviate from normal in their capacity to form the peptide bond of PAH, nor does their response to the added nutrients we studied seem abnormal.

#### SUMMARY

1 No significant difference in rate of formation of PAH, and in  $Q_{O_2}$  of liver and kidney slices from rats, has been found between unburned and burned animals at the height of the nitrogen loss in the urine.

2 Para-amino benzoic acid with and without added glycine significantly depresses the  $Q_{O_2}$  of liver slices from both burned and unburned animals.

3 Glucose reduces the  $Q_{O_2}$  of liver slices from unburned animals, and lactate significantly increases it in burned animals, but no significant difference is found between burned and unburned animals.

five kidney slices from each animal being set up in Warburg flasks in oxygen. Prior to burning, the animals were on a stock rat cake diet. Burning was carried out as described by Croft and Peters [1945], the experimental and control animals being anaesthetized with ether. Rats were sacrificed three days after burning, and during the intervening period the animals were tube-fed on a non-fat diet, previously described [Lathe and Peters, 1949]. Krebs-Ringer solution [Umbreit *et al.*, 1945], buffered with phosphate at pH 7.4 and enriched with 0.01 M glycine and 0.00102 M PAB, was used as a medium. Slices were cut with the Stadie apparatus. The flasks were flushed with pure oxygen for 5 minutes before being equilibrated in the bath at 37° C for 15 minutes. In the first series of experiments at the end of a two-hour incubation, each flask was analysed for PAH according to Cohen and McGilvery [1946].

The expression  $Q_{PAH}$  refers to the microlitres of PAH\* formed per hour, and is referred to the final nitrogen content (in mg) of the slices.

In the second series of experiments oxygen consumption was determined over an 80-minute interval, in duplicate upon liver slices.

### RESULTS

The  $Q_{PAH}$  (N) of liver and kidney slices are given in Table I. It may be calculated that a difference of approximately 25 per cent would have been required to be significant. No significant difference

TABLE I— $Q_{PAH}$  (N) AND  $Q_{O_2}$  OF LIVER AND KIDNEY SLICES, FIVE ANIMALS IN EACH GROUP, CONTROLS AND BURNED

	Liver		Kidney	
	Controls	Burned	Controls	Burned
$Q_{PAH}$ (N)	4.28 ± 507	4.03 ± 121	13.40 ± 943	12.32 ± 923
Difference		25 ± 52		1.08 ± 1.32
$Q_{O_2}$	65.56 ± 1.90	70.80 ± 1.44	123 ± 2.43	119 ± 6.7
Difference		5.24 ± 2.38*		4 ± 7

\*  $P = ca. 0.5$

was found in either tissue. The  $Q_{O_2}$  of liver and kidney slices over a two-hour period is also given in Table I. In the case of liver the difference is of marginal significance, while in kidney it is not significant. It was noted in these experiments that the oxygen consumption of liver was well maintained, while that of kidney dropped off to about 70 per cent of the initial value by the end of the two hours. In this respect experimental and control animals behaved alike.

In the second series of experiments the oxygen consumption of liver slices was determined in various substrates. By comparing the  $Q_{O_2}$  in a Krebs-Ringer medium with that when a substrate was

\* 22.4 litres PAB ≡ 1 mole



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